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(54) Title: GLYCOSAMINOGLYCAN-DNASE COMBINATION THERAPY

(57) Abstract: A method of improving the efficacy of the treatment with a DNase of a subject having a disorder characterised by the presence of endogenous extracellular DNA, comprises : (a) administering to the subject a glycosaminoglycan or a physiologically acceptable salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd to the subject; and (b) administering DNase to the subject.

GLYCOSAMINOGLYCAN-DNASE COMBINATION THERAPY

Field of Invention

The present invention relates to DNases and in particular to the use of DNases in the treatment of disorders characterised by the presence of endogenous extracellular DNA.

Background of Invention

DNases are a group of phosphodiesterase enzymes capable of hydrolysing DNA. They act in a non-specific manner, as they do not require the presence of a specific sequence in the target molecule and are capable of extensively degrading DNA molecules cleaving them multiple times. The two main types of DNases are DNase I and DNase II type enzymes. There are also DNase type III enzymes. DNase I requires cations to be active and has a pH optimum of near neutral. It hydrolyses DNA to produce 5'-phosphate nucleotides. DNase II can be activated by divalent cations and has an acidic pH optimum. It hydrolyses DNA to produce 3'-phosphate nucleotides.

Clinically DNases, and in particular DNase I, are mainly thought useful in the treatment of respiratory disorders and are presently used to treat cystic fibrosis. It has been suggested that DNases may also be useful in the treatment of a number of other conditions including those involving enclosed inflammatory sites and autoimmune disorders such as Systemic Lupus Erythematosus (SLE). The respiratory disorders which DNase is thought to be useful in treating are characterised by an increase in mucus viscosity due to the presence of human and bacterial genomic DNA.

Mucus is a complex mixture of salts, water, glycoproteins, proteoglycans and proteins that cleanses, lubricates and protects many passageways in the body, including those in the lungs. However, the presence of genomic DNA and in particular long strands of genomic DNA, increases mucus viscosity to the point where mucociliary clearance is compromised. The viscous mucus is not cleared properly by the normal mechanisms and blocks or obstructs the airways of the lung, thereby restricting airflow. It may also promote the occurrence of infections. Overall

the effect may be fatal or dehabilitating.

It is thought that the viscous genomic DNA in these respiratory conditions mainly originates from infiltrating inflammatory cells such as neutrophils. These cells are believed to undergo necrosis, rather than the more controlled process of apoptosis, releasing large amounts of gelatinous genomic DNA into the mucus. The release of other polymers, such as actin, from the necrosing cells may further increase the viscosity of the mucus.

Cystic fibrosis is one of the main respiratory disorders where abnormally increased mucus production and the presence of DNA in the mucus play an important role. Cystic fibrosis is an autosomal recessive inherited disorder caused by mutation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. It is the most common inherited disorder in individuals of European descent and also occurs in other populations, but normally with a lower incidence. Patients with cystic fibrosis typically display difficulty in breathing, excessive salt loss during sweating, and incomplete digestion and absorption of food. The disease may also affect male fertility.

CFTR is a chloride channel and is present in mucus secreting cells. Chloride ions are released into the mucus via the CFTR channel. This raises the osmolarity of the mucus and results in the passage of water from the cells into the mucus, thinning it and helping to ensure that it has the correct viscosity. In cystic fibrosis patients, due to the defect in CFTR, chloride ions are not effectively transported into the mucus and hence the mucus is more viscous. The uptake of sodium, normally restricted by CTFR activity, is increased and, with it, water uptake leading to dehydrated condensed secretions that promote bacterial infection and hence inflammation. The number of white blood cells, and in particular neutrophils, in the mucus in the lung of cystic fibrosis patients is also greatly increased and necrosis of these cells releases genomic DNA which further increases mucus viscosity.

Cystic fibrosis is normally fatal, although life expectancy for afflicted individuals is increasing due to improved treatments. In the 1960's on average cystic fibrosis sufferers had a life expectancy of less than ten years, whereas now average life expectancy is approaching thirty years.

DNases, and in particular the recombinant human DNase I commonly used in the treatment of cystic fibrosis, are expensive. Furthermore, although DNases are presently used to treat cystic fibrosis patients, some 50 to 70% of cystic fibrosis patients show only minimal or no benefit following treatment.

Summary of the Invention

The present invention is based on the finding that glycosaminoglycans which have an average molecular weight of from 8 to 40kd can increase the activity of a DNase and in particular DNase I. The glycosaminoglycan itself does not cleave DNA, rather it increases the ability of DNase to do so. This unexpected synergistic effect means that less DNase is necessary to achieve the same effect and also that higher levels of total activity may be achievable in a system using the same amount of DNase.

The higher level of activity achievable with the same amount of enzyme may mean that conditions or individuals previously refractory to treatment with DNases, or which derived minimal benefit from treatment, may now be treatable. It may also mean that less DNase has to be used which is in particular important in applications where recombinant DNases are employed as these enzymes are expensive.

Accordingly, the present invention provides the use of a glycosaminoglycan or a physiologically acceptable salt thereof, in the manufacture of a medicament for treating a subject with a disorder characterised by the presence of endogenous extracellular DNA wherein the subject is also being treated with a DNase and the glycosaminoglycan or salt has an average molecular weight of from 8 to 40 kd

The present invention also provides the use of a DNase in the manufacture of a medicament for treating a subject with a disorder characterised by the presence of endogenous extracellular DNA wherein the subject is also being treated with a glycosaminoglycan or a physiologically acceptable salt thereof, and the glycosaminoglycan or salt has an average molecular weight of from 8 to 40kd.

The present invention further provides for a composition comprising a glycosaminoglycan or a salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40 kd and a DNAse.

The present invention also provides for:

- products comprising a glycosaminoglycan or a physiologically acceptable salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd, and a DNase for simultaneous, separate or sequential use in the treatment of a disorder characterised by the presence of endogenous extracellular DNA in the subject to be treated;

- a method of improving the efficacy of the treatment with a DNase of a subject having a disorder characterised by the presence of endogenous extracellular DNA, which methods comprises the steps of:

  (a) administering to said subject a glycosaminoglycan or a physiologically acceptable salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd to the subject; and

  (b) administering to said subject a DNase;

- an agent for treating a subject having a disorder characterised by the presence of endogenous extracellular DNA, the agent comprising a glycosaminoglycan or a physiologically acceptable salt thereof, wherein the glycosaminoglycan or salt has an average molecular weight of from 8 to 40kd and the said subject is being treated with a DNase; and

- an agent for treating a subject having a disorder characterised by the presence of endogenous extracellular DNA, the agent comprising a DNase and the said subject being treated with a glycosaminoglycan or a physiologically acceptable salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd.

The present invention further provides a kit comprising:

- a glycosaminoglycan or a physiologically acceptable salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd;

- instructions for the sequential, simultaneous or separate administration of the glycosaminoglycan or salt to a subject suffering from a disorder characterised by the presence of endogenous extracellular DNA and being treated with a DNase; and

- packaging.

The invention also provides a kit comprising:

- a DNase;
- instructions for the sequential, simultaneous or separate administration of the glycosaminoglycan or salt to a subject suffering from a disorder characterised by the presence of endogenous extracellular DNA and being treated with a glycosaminoglycan or a physiologically acceptable salt thereof, having an average molecular weight of from 8 to 40kd; and
- packaging.

The invention also provides:

- a nebuliser or other liquid aerosol device, dry powder inhaler or metered dose inhaler comprising a composition according to the invention; and
- use of a glycosaminoglycan or a physiologically acceptable salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd to increase the activity of a DNase *in vitro*.

Brief Description of the Figures

Figure 1 shows the effect of unfractionated heparin on the degradation of DNA by DNase. The Y axis shows DNA concentration after incubation with DNase for 1 hour. The X axis shows the DNase concentration employed. Results are provided for incubations carried out in the presence of heparin (□) and in the absence of heparin (♦).

Figure 2 shows the effect of unfractionated heparin on the degradation of DNA by DNase as determined by agarose gel electrophoresis. The top panel shows the results of incubation of genomic DNA with DNase in the absence of heparin, whereas the bottom panel shows the results when the same digestions were performed in the presence of heparin. Lanes 1 to 12 are as follows: lanes 1 & 2 - DNA alone; lanes 3 & 4 - DNA + DNase (5 minute incubation); lanes 5 & 6 – DNA + DNase (15 minute incubation); lanes 7 & 8 - DNA + DNase (30 minute incubation); lanes 9 & 10 - DNA + DNase (45 minute incubation); lanes 11 & 12 -

DNA + DNase (60 minute incubation).

Figure 3 shows the effect of unfractionated heparin on the degradation of DNA by DNase as determined by atomic force microscopy (AFM). Panel A shows the results for DNA incubated on its own. Panel B shows the results for DNA incubated with DNase. Panel C shows the results for DNA incubated with DNase and 1 microgram per ml of heparin. Panel D shows the results for DNA incubated with DNase and 10 micrograms per ml of heparin.

Figure 4 shows the effect of various glycosaminoglycans (GAGs) on DNase activity. The Y axis shows the concentration of DNA remaining after one hour incubation with DNase and the GAG, whilst the X axis shows the concentration of GAG employed. Results are shown for (A) heparin; (B) a mixture chondroitin sulphates A and C; (C) heparan sulphate; (D) LMW heparin; and (E) dextran sulphate (which is a non-GAG control).

Figure 5 shows the effect of a mixture of chondroitin sulphates A and C on DNase efficacy in cystic fibrosis sputum as assessed using a barrier assay involving measurement of the transport of fluorescent microspheres. Results are shown for a variety of chondroitin sulphate concentrations.

Figure 6 shows the effect of a mixture of chondroitin sulphates A and C on the degradation of DNA by DNase as determined by atomic force microscopy (AFM). Panel A shows the results obtained in a DNA control with no DNase or chondroitin sulphate. Panel B shows the results for DNA incubated with DNase alone. Panel C shows the results for DNA incubated with both DNase and chondroitin sulphate.

Brief Description of the Sequences

SEQ ID NO: 1 provides the nucleotide and amino acid sequence for human DNase 1.

SEQ ID NO: 2 provides the amino acid sequence for human DNase 1.

Detailed Description of the Invention

The present invention is concerned with administration, to a subject having a disorder characterised by the presence of endogenous extracellular DNA, of a glycosaminoglycan or a physiologically acceptable salt thereof, to potentiate the activity of a DNase that is administered to the subject to treat the disorder. The activity of the DNase is thus enhanced. A synergistic effect can be observed. In an especially preferred embodiment of the invention the glycosaminoglycan employed will be a heparin, a derivative thereof or a physiologically acceptable salt of either thereof. In another especially preferred embodiment of the invention the DNase employed will be a DNase I. In a further especially preferred embodiment the glycosaminoglycan will be administered intranasally and/or via inhalation.

Synergy of glycosaminoglycan with DNAse

Unexpectedly glycosaminoglycans with an average molecular weight of from 8 to 40 kd are capable of increasing the activity of DNase. That is that the same molar amount of enzyme has increased activity in the presence of glycosaminoglycan compared to in the absence of glycosaminoglycan. Glycosaminoglycan alone have no, or negligible, DNA hydrolytic activity thus the increase in activity is specifically due to an increase in the enzyme's activity in the presence of glycosaminoglycan.

The rate of DNA digestion i.e. the amount of DNA digested per unit time may be increased by from one, two, three, ten, twenty or more fold by the addition of the glycosaminoglycan. The enhancement in DNase activity may typically be from 5% to 5000%, preferably from 50 to 2500%, more preferably from 75 to 1000%, still more preferably from 100 to 1000%, yet more preferably from 250 to 1000% and even more preferably from 500 to 1000%. These enhancements will typically refer to the amount of DNA the DNase can degrade in a given time and preferably to the activity of the enzyme as expressed in Kunitz units or alternatively Dornase units.

One Kunitz unit of DNase will produce a delta A260 of 0.001 per minute per ml at pH 5.0 at 25°C, using DNA as substrate, with $[Mg^{2+}] = 4.2$ mM. Preferably the

DNA used to assess the DNAse activity will be calf thymus or salmon sperm genomic DNA. A Dornase unit is defined as the amount of enzyme that will cause a decrease of 1.0 relative viscosity unit in a solution of highly polymerised DNA from the original viscosity of 4.0 in 10 minutes at 37°C.

The presence of glycosaminoglycan may effectively increase the number of units of DNase enzyme activity present by more than one fold, double, treble, five fold, ten fold, twenty fold or more. Typically, the presence of glycosaminoglycan may effectively reduce the molar amount of DNase necessary to achieve the same activity by a half, a quarter, a fifth, a tenth or more. The glycosaminoglycan may ensure a more complete digestion of DNA in a given time by the same amount of DNase thus the average, or main fragment size, present may typically be twice, three, five, ten, twenty, fifty or more times longer in the absence than in the presence of glycosaminoglycan following incubation for an equivalent amount of time.

A number of methods are described herein for assaying for DNase activity. Any of these may be used to assess DNase activity. In particular a fluorescence based assay using, for example, Hoechst Stain may be used such as that described in Labarce & Paiden, 1980, Anal. Biochem., 102:344-352 to assay for DNA hydrolysis. Gel electrophoresis may be used to assess DNA fragment size.

The glycosaminoglycan or salt thereof is typically provided in an amount that produces a synergistic effect on DNase activity. Thus one or more of the values given herein for increase in activity may be achieved. The ratio of DNase to glycosaminoglycan, or salt by weight or alternatively by units may, for example, be from 1:50,000 to 1000:1, preferably from 1: 10,000 to 100:1, more preferably from 1: 5,000 to 50:1, still more preferably be from 1:1,000 to 25:1. The ratio may, for example, be from 1:500 to 1:20, preferably be from 1:150 to 1:5, more preferably be from 1:50 to 1:2 and even more preferably be from 1:10 to 1:1. The two may, for example, be in equal amounts or the DNase may be two fold, five fold, ten fold or more excess or alternatively the glycosaminoglycan may be present in similar excess.

DNAses

Any suitable DNase may be used in the present invention. The DNase will

most preferably be a DNase I (EC 3.1.21.1). It may, however, in some embodiments be a DNase II (EC 3.1.21.1). DNases occur in a number of species and any DNase capable of cleaving DNA may be used in the invention. The DNase may be from an animal source such as of bovine or porcine origin. It may be of plant, fungal, or microbial origin. However, typically and most preferably the DNase is of human origin and is preferably a recombinant human DNase. Commercially available DNase preparations such as Dornase™ and Pulmozyme™ may be used in embodiments of the invention.

The DNase will have DNA hydrolytic activity, for example in the case of DNase I it may hydrolyse DNA to give 5'-phosphate nucleotides and in the case of DNase II it may hydrolyse DNA to give 3' phosphate nucleotides. A fluorescence based assay using, for example, Hoechst Stain may be used such as that detected in Labarce & Paiden, 1980, Anal. Biochem., 102:344-352 to assay for DNA hydrolysis. Hydrolytic activity may be assessed in a variety of ways known in the art including analytical polyacrylamide and agarose gel electrophoresis, hyperchromicity assay (Kunitz, J. Gen. Physiol. 33:349-362 (1950); Kunitz, J. Gen. Physiol. 33:363-377 (1950)) or methyl green assay (Kurnick, Arch. Biochem. 29:41-53 (1950); Sinicropi, et al., Anal. Biochem. 222:351-358 (1994)). The breakdown of DNA molecules from high to lower molecular weight forms may be monitored preferably by techniques such as agarose gel electrophoresis. Control experiments in the absence of the enzyme and/or in the presence of a protein known to possess DNase activity may be performed.

The DNase will preferably display mucolytic activity for samples of mucus containing DNA. Mucolytic activity refers to the reduction of viscoelasticity (viscosity) of mucus. Mucolytic activity may be determined by any of several different methods known in the art, including sputum compaction assay (see for example WO 94/10567) assays using a torsion pendulum (Janmey, J. Biochem. Biophys. Methods 22:41-53 1991) or other suitable rheological methodologies.

DNases are known to be prone to deamidation. Asparagine residues and in particular the asparagine at amino acid positions 7 and 74 of the mature human DNase I are prone to deamidation. This process converts the asparagine residues in

question to aspartic acid or iso-aspartate residues. Deamidation reduces the activity of the enzyme and this is particularly the case for deamidation at the asparagine at amino acid position 74 of mature human DNase I.

Techniques are available for removing deamidated forms of the enzymes to leave the amidated forms and these may be employed to prepare DNases for use in the invention. These techniques may include tentacle cation exchange (TCX) or affinity purification using DNA to purify the amidated forms of the enzyme which are still capable of binding DNA.

A DNase preparation for use in the invention may typically comprise from 85 to 100%, preferably from 90 to 100%, more preferably from 95 to 100% and even more preferably from 99 to 100% amidated, or partially amidated, enzyme by weight. In particular these figures refer to the amount of wholly amidated enzyme i.e. with all of the residues which are naturally amidated being amidated. They will typically have more than 95%, preferably more than 99% and even more preferably more than 99.9% of the DNase in an amidated form. In particular, these values refer to values at the time of production or to their values from one month to a year, preferably from two to six months and more preferably from three to four months after production. They may refer to the value during any point of the shelf-life of the product.

It is known that certain surfaces promote deamidation of DNase and hence that the pharmaceutical compositions, preparations and medicaments of the invention are preferably not stored in contact with glass. They may, however, be stored in glass containers which are coated with a non-glass layer so that glass is not in contact with the DNase. Alternatively they may be provided in plastic containers or other suitable non-glass containers.

Variants of naturally occurring or known DNases may be used in the invention. Thus the term DNase encompasses such variants. The term "variants" refers to polypeptides which have the same essential character or basic biological functionality as DNase. The essential character of a DNase is phosphodiesterase activity and the ability to hydrolyse DNA. Assays for measuring DNA cleavage are described herein and these may be used to determine whether a variant has hydrolytic activity.

SEQ ID NO: 2 provides the amino acid sequence for human DNase 1 and variants of this sequence are encompassed in the invention. Typically, polypeptides with more than about 65% identity, preferably at least 80% or at least 90% and particularly preferably at least 95%, at least 97% or at least 99% identity, with the amino acid sequence of SEQ ID NO: 2 which retain DNA hydrolytic activity, are considered as variants which may be employed in the invention. Such variants may include allelic variants and the deletion, modification or addition of single amino acids or groups of amino acids within the protein sequence, as long as the peptide maintains DNA hydrolytic activity.

Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions may be introduced. The modified polypeptide will retain DNase activity. Conservative substitutions may be made, for example according to the following Table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Shorter variants are within the scope of the invention. For example, a peptide of at least 20 amino acids or up to 50, 60, 70, 80, 100, 150 or 200 amino acids in length is considered to fall within the scope of the invention as long as they demonstrate DNA hydrolytic activity. In particular, but not exclusively, this aspect of

the invention encompasses the situation when the protein is a fragment of the complete protein sequence of SEQ ID NO:2 and may represent a DNA-binding and hydrolytic region in particular include the active site.

A variety of programs may be used to calculate percentage homology. The UWGCG Package provides the BESTFIT program which can be used to calculate homology (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* **12**, p387-395). The PILEUP and BLAST algorithms can be used to calculate homology or line up sequences (typically on their default settings), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S, F *et al* (1990) J Mol Biol 215:403-10.

Software for performing BLAST analyses is publicly available through the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al*, supra). These initial neighbourhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci.* USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm

is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

In one embodiment of the invention the DNase employed may actually be a variant which has reduced affinity for actin compared to the DNase it is derived from. These variants may, for example, be used where actin is present in addition to the DNA and preferably where the disorder to be treated is a respiratory disorder where actin is present in the mucus.

The variant may have from 0 to 50%, preferably from 0 to 25 %, more preferably from 0 to 15 % and even more preferably from 0 to 5% of the affinity of the unmodified DNase it is derived from for actin and preferably of that of human DNase I. The affinity of the variant for actin may be reduced from by from 1 to 1000 fold, preferably from 5 to 500 fold, more preferably by from 10 to 100 fold and even more preferably by from 20 to 50 fold. These figures may refer to affinity for polymeric or monomeric action or both. The variant may lack the ability to bind to specifically actin altogether. Variants with reduced activity for actin will, however, retain some, or all, of their hydrolytic ability and their ability to bind and cleave DNA.

Amino acid changes may be made to a DNase to introduce a glycosylation site at, or near, to an actin binding site of the DNase to reduce the affinity of the variant for actin. Regions or residues responsible for actin binding may be deleted or substituted with alternative amino acids. Insertions or modifications may be made close to actin binding sites to disrupt their function. Preferred DNase variants with reduced affinity for actin are detailed in US Patent 6,348,343 and any of the DNase variants detailed therein with reduced affinity for actin, but which retain DNA hydrolytic activity, may be employed in the present invention. Equivalents modifications to those described in US patent 6,348,343 may be made to other DNases including DNase II. The reduction in the ability to bind actin can then be

determined to check affinity for actin is reduced, whilst DNA hydrolytic activity is retained.

The ability of a variant to bind actin may be monitored by techniques known in the art such as those detailed in Mannherz, *et al.*, Eur. J. Biochem. 104:367-379 (1980). The relative binding affinities of different DNases may be determined by measuring the binding of the DNases to immobilised actin in an ELISA assay or by comparing the DNA-hydrolytic activity of the DNases in the presence and absence of actin.

The sequence of the DNase may be modified so that it has extended half-life. For example the sequence of the enzyme may be changed to remove recognition sequences for certain proteases and in particular those derived from inflammatory cells present in the lung or other systems where the invention will be employed.

The DNase employed in the invention may also be chemically modified to alter its properties such as, for example, its biological half-life. To achieve this covalent modifications may be introduced by reacting targeted amino acid residues of the native or variant DNase with an organic derivatising agent that is capable of reacting with selected amino acid side-chains or N- or C-terminal residues. Suitable derivatising agents and methods are well known in the art.

Residues which in particular may be derivatised include cysteinyl residues (most commonly by reaction with α-haloacetates), histidyl residues (by reaction with diethylpyrocarbonate at pH 5.5-7.0), lysinyl and amino terminal residues (by reaction with succinic or other carboxylic acid anhydrides), arginyl residues (by reaction with reagents such as phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin). Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides or may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions. The covalent attachment of agents such as polyethylene glycol (PEG) or human serum albumin to the DNases may reduce their immunogenicity and/or toxicity of the variant and/or prolong its half-life and hence may be used in the invention.

In some embodiments of the invention the DNase may be directly conjugated to the glycosaminoglycan or joined through an intermediate molecule.

Glycosaminoglycans

The medicaments and methods of the invention employ glycosaminoglycans. Glycosaminoglycans are linear heteropolysaccharides possessing characteristic disaccharide repeat sequences that are typically highly N-and O-sulpated at D-glucosamine, galactosamine and uronic acid residues. These sulphate moieties introduce a high degree of negative charge along the glycosaminoglycan polymer chain and add to the heterogeneity of these macromolecules.

Any suitable glycosaminoglycan may be employed in the invention. Preferably, the glycosaminoglycan will be in a form suitable for administration intranasally, via inhalation, and/or via installation as typically the medicaments of the invention will be administered by such routes. Glycosaminoglycans and glycosaminoglycan salts suitable for use in the present invention will have an average molecular weight of from 8 to 40 kd, preferably from 10 to 30 kd, more preferably from 12 to 20 kd. In particular, the glycosaminoglycan or salt may have an average molecular weight of from 14 to 18kd, preferably from 15 to 17 kd and more preferably from 16 to 17 kd. In some embodiments all, or substantially all, of the glycosaminoglycan molecules or glycosaminoglycan salt molecules will have a molecular weight falling within the ranges specified above. Thus from 50 to 100%, preferably from 75 to 100%, more preferably from 90 to 100%, still more preferably 95 to 100% of the molecules may have such a molecular weight. In some cases at least 95%, preferably 97.5%, more preferably 99%, still more preferably 99.5% and even more preferably 99.9% may have a molecular weight falling within the range. The glycosaminoglycan or salt may be present in a range of molecular weight sizes and typically the most commonly occurring molecular weight size will fall within one of the above specified molecular weight ranges.

Preferably, the glycosaminoglycan employed in the invention will be any of chondroitin sulphates A to E, heparin, heparin sulfate, heparan, heparan sulphate, hyaluronic acid, keratan sulphate, a derivative of any thereof or a mixture or any two thereof. Chondroitin sulphate B is sometimes referred to as dermatan sulphate. In a more preferred embodiment of the invention the glycosaminoglycan will be any of

chondroitin sulphates A, C, D or E, heparin, heparin sulfate, heparan, heparan sulphate, hyaluronic acid, keratan sulphate, a derivative of any thereof or a mixture of any two thereof. In a particularly preferred embodiment the glycosaminoglycan will be chondroitin sulphate A, chondroitin sulphate C, heparin, heparin sulfate, heparan, heparan sulphate, a derivative of any thereof or a mixture of any two thereof. More preferably, the glycosaminoglycan will be chondroitin sulphate A, chondroitin sulphate C, heparin, a derivative of any thereof or a mixture of any two thereof. In an even more preferred embodiment of the invention the glycoaminoglycan will be heparin or a derivative thereof. In some embodiments of the invention the glycosaminoglycan employed will be a mixture of more than two glycosaminoglycans from one of the above mentioned groups, such as a mixture of three, four or five of the glycosaminoglycans.

In embodiments of the invention where a mixture of two glycosaminoglycans is employed the two may, for example, be present in the ratio 1:1, 1:2, 1:4, 1:10 or 1:100. The ratio may be 90:10, 80:20, 70:30, or 60:40. Any suitable ratio may be employed and either glycosaminoglycan may be at the higher concentration. The ratio may be the same as the ratio in which the two are isolated when they are recovered from a common tissue using standard techniques. In a preferred embodiment of the invention a mixture of chondroitins A and C will be employed and in particular at a ratio of 80:20, preferably 75:25 and even more preferably 70:30 with chondroitin sulphate A being present at the higher level.

Typically, the glycosaminoglycan will not have been subjected to fragmentation to reduce its molecular weight. Usually, the glycosaminoglycan will not have been subjected to depolymerisation, such as by chemical or enzymatic means, to reduce its molecular weight. The average number of saccharide units in the polysaccharide chains of the glycosaminoglycan may typically be from 18 to 100, preferably from 30 to 80, more preferably from 40 to 60 and still more preferably from 5 to 60 units.

The glycosaminoglycan may be any suitable commercially available glycosaminoglycan and may, for example, be an unfractionated glycosaminoglycan. The glycosaminoglycan will have typically been isolated from a natural sources such

as from an animal. In some cases, the glycosaminoglycan may have been synthesised rather than be a naturally occurring molecule.

In some cases the glycosaminoglycan may have been isolated from an animal, and in particular from animal tissues such as those of pigs or cattle. The glycosaminoglycan may have been obtained from tissues such as the lung, liver, or gut of an animal and in particular from beef lung or pork intestinal mucosa. The glycosaminoglycan may have been obtained from the skin of such an organism.

In some embodiments, the glycosaminoglycan may have been isolated from a cartilagenous fish or other sea or freshwater organism. In some cases the glycosaminoglycan may have been isolated from a shark or squid and in particular from the cartilage of such an organism. The glycosaminoglycan may have been isolated from a sturgeon and in particular from a sturgeon notochord.

One of the specific glycosaminoglycans mentioned above may have been modified to generate a derivative of the glycosaminoglycan. Such derivatives may be used in the invention as long as they retain the ability to increase DNase activity. Thus in the presence of the derivative a given molar amount of DNase will display a higher level of activity than in the absence of the derivative. Thus in the case of heparin, the heparin may have been subjected to O-desulphation such as at least at the 2-O and 3-O positions. The same or equivalent modifications may be made to other glycosaminoglycans to generate derivatives for use in the present invention.

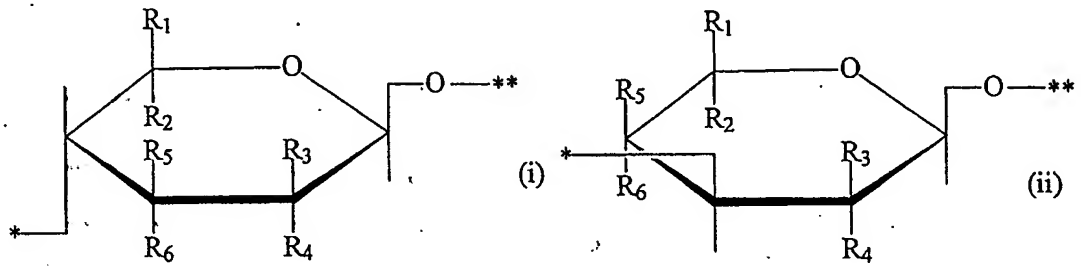
The glycosaminoglycan may have been subjected to acetylation, deacetylation, oxidation and/or decarboxylation such as, for example, periodate oxidation to generate a derivative. Heparinoids may be used in the invention.

Typically, the active compound used in the present invention comprises a glycosaminoglycan or a physiologically acceptable salt thereof comprising repeating disaccharide units of general formula (1):

-[A-B]- (1)

wherein:

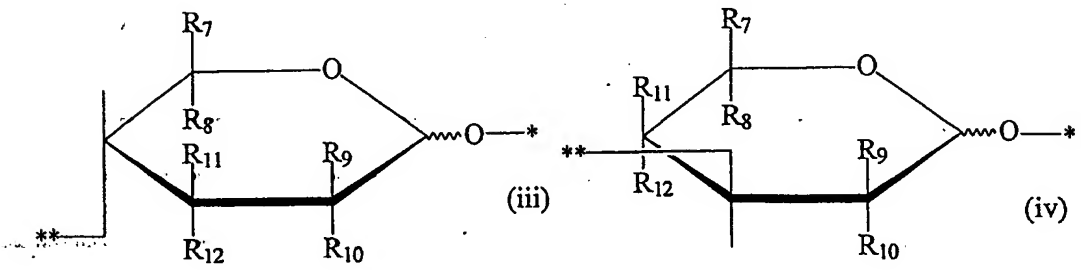
each A is the same or different and represents a moiety of formula (i) or (ii)



wherein:

- one of R_1 and R_2 is hydrogen, and the other is $-CO_2H$, $-SO_3H$ or $-CH_2OR$ wherein R is hydrogen or $-SO_3H$;
- one of R_3 and R_4 is hydrogen, and the other is -OR wherein R is hydrogen or $-SO_3H$;
- one of R_5 and R_6 is hydrogen, and the other is -OH;
- * represents a direct bond to an adjacent hydrogen atom or B moiety; and
- ** represents a direct bond to an adjacent B moiety;

each B is the same or different and represents a moiety of formula (iii) or (iv);



wherein:

- one of R_7 and R_8 is hydrogen and the other is $-CH_2OH$ or $-CH_2OSO_3H$;
- one of R_9 and R_{10} is hydrogen and the other is -NHAc, $-NH_2$ or $-NHSO_3H$;
- one of R_{11} and R_{12} is hydrogen and the other is -OH or $-OSO_3H$;
- * represents a direct bond to a hydrogen atom or an adjacent A moiety;
- ** represents a direct bond to an adjacent A moiety; and

- indicates a bond in either stereochemical orientation;

or a physiologically acceptable salt thereof.

The formulae herein adopt standard practice in depicting sugars. According to this practice, the formulae include vertical lines through each of the cyclic carbon atoms. This does not, of course, mean that methyl groups are attached at each position, or that methylene groups are present as part of the link between adjacent cyclic moieties.

Preferably, each A moiety in the glycosaminoglycan of general formula (1) is the same. Preferably, each B moiety in the glycosaminoglycan of general formula (1) is the same.

Preferably, each A moiety in the glycosaminoglycan of general formula (1) is a moiety of general formula (i).

Typically, one of R_1 and R_2 is hydrogen, and the other represents $-CO_2H$ or $-CH_2OR$, wherein R is hydrogen or $-SO_3H$. Preferably, one of R_1 and R_2 is hydrogen and the other represents $-CO_2H$.

Typically, R_3 is hydrogen and R_4 is -OR, wherein R represents hydrogen or $-SO_3H$.

Typically R_5 is -OH and R_6 is hydrogen.

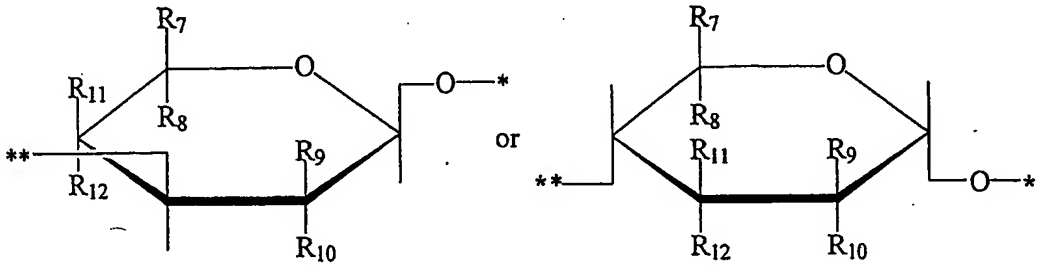
Typically each A is the same or different and represents a moiety of formula (i).

Typically R_7 is $-CH_2OH$ or $-CH_2OSO_3H$ and R_8 is hydrogen.

Typically R_9 is hydrogen and R_{10} is -NHAc, $-NH_2$ or $-NHSO_3H$.

Typically R_{11} is $-OSO_3H$ or -OH and R_{12} is hydrogen.

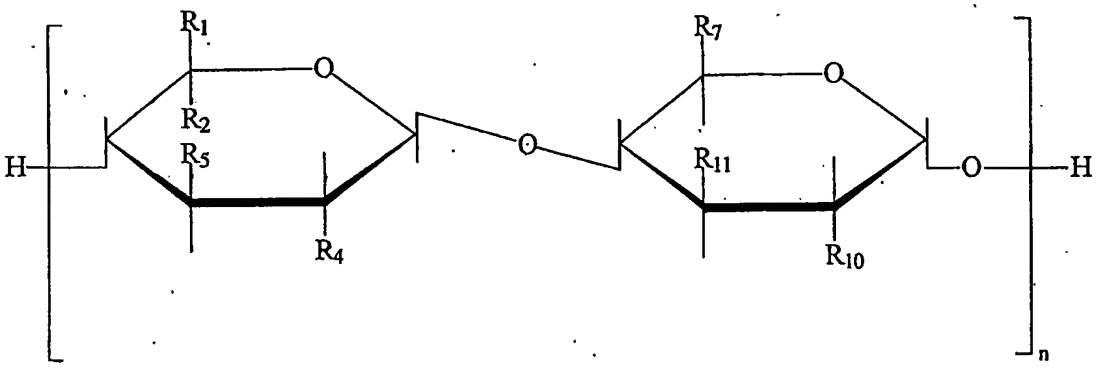
Typically each B is the same or different and represents



wherein R_7, R_8, R_9, R_{10}, R_{11}, R_{12} * and ** are as described above.

Preferably R_1 is not hydrogen in an A moiety which is adjacent to a moiety (iv) in which R_{12} is hydrogen.

Typically the glycosaminoglycan of general formula (1) is a glycosaminoglycan of general formula (2)

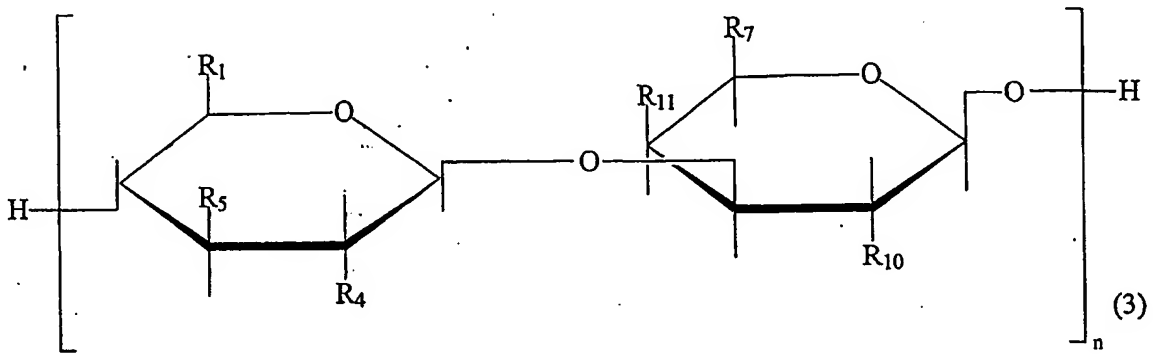


(2)

wherein:

- one of R_1 and R_2 is hydrogen and the other is $-CO_2H$;
- R_4 is -OH or $-OSO_3H$;
- R_5 is -OH;
- R_7 is $-CH_2OH$ or $-CH_2OSO_3H$;
- R_{10} is $-NH_2$, $-NHSO_3H$ or -NHAc; and
- R_{11} is $-OSO_3H$ or -OH.

Typically the glycosaminoglycan of general formula (1) is a glycosaminoglycan of general formula (3)



(3)

wherein:

- R_1 is $-CO_2H$;
- R_4 is -OH;
- R_5 is -OH;
- R_7 is $-CH_2OH$ or $-CH_2OSO_3H$;
- R_{10} is -NHAc; and
- R_{11} is -OH or $-OSO_3H$.

Any suitable physiologically acceptable glycosaminoglycan salt may be employed in the invention and in particular a metallic salt, for example a sodium salt, an alkali metal or an alkaline earth metal salt. Other salts include calcium, lithium and zinc salts. Ammonium salts may also be used. The salt may be a sodium glycosaminoglycanate or glycosaminoglycan sulphate. Salts of derivatives of specific glycosaminoglycans mentioned herein may also be used in the invention. In the present application where mention of a glycosaminoglycan is made, such mention also includes physiologically acceptable salts thereof.

Typically a physiologically acceptable salt is a salt with a physiologically acceptable acid or base. Preferred salts are salts with physiologically acceptable bases. Typically, such salts are compounds wherein the acidic hydrogen atom of a $-CO_2H$ and/or $-OSO_3H$ group is replaced with a cation, for example an alkali metal (e.g. sodium or potassium) or alkali earth metal (e.g. calcium or magnesium) cation. Such salts can be prepared, for example, by reaction with an appropriate hydroxide.

The number of disaccharide units present in the glycosaminoglycan or salt

thereof employed in the invention will be such that the molecular weight of the glycosaminoglycan or salt is from 8 to 40 kd, preferably from 10 to 30 kd, more preferably from 12 to 20 kd. In particular, it may be such that the glycosaminoglycan has a molecular weight of from 14 to 18 kd, preferably from 15 to 17 kd and more preferably from 16 to 17 kd. The number of disaccharide units present in the glycosaminoglycan may be represented by the number n, where n is any integer such that the glycosaminoglycan has a molecular weight falling within any of the above mentioned molecular weight ranges.

Thus the glycosaminoglycan or salt employed may be represented by the general formula:

$$H\text{-}[A\text{-}B]_n\text{-}H$$

wherein -A-B is any of the disaccharides mentioned above and n is an integer such that the glycosaminoglycan or salt thereof has a molecular weight falling within the above specified molecular weight ranges. The value of n may be, for example, from 30 to 55 and more preferably from 35 to 50.

The glycosaminoglycan employed in the invention will typically comprise more than one length chain. Hence n for some of the glycosaminoglycan chains present may be a lower or higher integer than an integer which, on its own, would give a chain of molecular weight size falling within one of the above specified ranges. Thus the average value of n of the glycosaminoglycans present in the medicaments of the invention may be any of the values specified for n herein and in particular a value of n which gives a molecular weight glycosaminoglycan or salt falling within one of the molecular weight ranges specified herein.

Particularly preferred salts for use in the invention are salts of formula:

$$H\text{-}[A\text{-}B]_n^{x-}\text{-}H \quad M^{x+}$$

wherein $x \leq 4n$ and M represents a physiologically acceptable cation or a mixture thereof. Most preferably, $x \leq 2n$.

In a particularly preferred embodiment of the invention the glycosaminoglycan employed will be a heparin, a derivative thereof or a physiologically acceptable salt thereof. Heparin is a naturally occurring mucopolysaccharide present in a variety of organs and tissues, particularly liver,

lung, and the large arteries. Heparin is a polymer of alternating α-D-glucosamine and hexuronate residues joined by (1,4) glycosidic linkages. When glycosaminoglycans are synthesised in nature, typically they are conjugated to a central protein core. However, preferably the glycosaminoglycans employed in the invention will lack such a central core. Typically, glycosaminoglycan preparations will lack a core and may be employed or, if present, the core can be removed. Commercially available preparations of glycosaminoglycans will usually lack the core and may be employed.

Heparin is clinically used as an anti-coagulant, where it is thought to exert its effects through interaction with anti-thrombin III (AT-III) and heparin co-factor II and other coagulation factors. Typically the heparin will retain some anticoagulant activity i.e. be able to increase clotting time in an individual. Thus preferably the heparin will be able to bind anti-thrombin III (AT-III) and/or heparin co-factor II (HCII) and hence inhibit clotting. Preferably it will be able to form a complex with AT-III, thrombin and a clotting factor. However, in some embodiments a heparin which lacks anti-coagulant activity or which has reduced anti-coagulant activity may also be employed. Thus the heparin may have been modified so that it has from 0 to 80%, preferably from 5 to 60%, more preferably from 10 to 40% and even more preferably from 10 to 30% of the activity of the unmodified form or in comparison to unmodified heparin. Other glycosaminoglycans, in particular dermatan sulphate, also possess anticoagulant activity. Preferably, therefore, the glycosaminoglycans and their derivatives employed will retain some anti-coagulant activity, as discussed above for heparin and its derivatives.

Subjects to be treated

The subject to be treated will typically have a disease or disorder characterised by the presence of endogenous extracellular DNA. That is there will be extracellular DNA present originating from the cells of the body of the subject. Typically the DNA will be present in one of the extracellular solutions or secretions of the body. Thus in preferred embodiments the DNA may be present in the lung including its airways. Typically this DNA will originate from the nucleus and hence

be genomic DNA

The extracellular DNA will typically be of high molecular weight. Thus it may be, for example, that it has an average fragment size, fragment size range or predominate fragment size in the range of from 100 bases to 1 megabase, preferably from 1 kb to 500 kb, more preferably from 5 kb to 250 kb in length, still more preferably from 25 kb to 100 kb and even more preferably from 25 kb to 50 kb in length. In embodiments of the invention where the condition to be treated is an autoimmune disorder such as SLE, the DNA may be of shorter length or may fall within the ranges specified above.

The DNA will typically be wholly, or partially free of, histones or will comprise regions which lack histones. It may be bound by cationic factors or factors with cationic groups such as inflammatory mediators and/or proteases. Thus for example it may be complexed or associated with elastase, cathepsins and/or IL-8.

The presence of the DNA will typically increase the viscosity of the solution or liquid it is present in. This will, in particular, be the case where the subject is suffering from a respiratory disorder where typically the presence of the DNA will increase the viscosity of mucus or other pulmonary secretions. It may also be the case where the object is to treat or help resolve an inflammatory site and in particular an enclosed inflammatory site in which there is extracellular DNA present.

Typically, the presence of DNA may increase the viscosity of the solution it is present in by from 10 to 5000%, preferably from 50 to 2500%, more preferably from 100 to 1000%, still more preferably from 200 to 800% and even more preferably from 400 to 600%. The viscosity of the solution may typically be doubled, tripled, quadrupled or increased by five fold or more in comparison to the viscosity of the solution in the absence of the DNA. Typically the viscosity of the DNA will be such that it may comprise such function in the subject such as, for example, lung function. It may cause airflow limitation and/or promote the occurrence of infections.

In one embodiment of the invention the subject may be suffering from inflammation and in particular inflammation where there is extracellular DNA at the inflammatory site. The main inflammatory site may, in particular, be enclosed either partially or totally. The site may contain or produce large amounts of pus or other

inflammatory material. Examples of conditions include abscesses, meningitis, peritonitis, sinusitis, otitis, periodontitis, pericarditis, pancreatitis, cholelithiasis, endocarditis and septic arthritis. The subject may have inflammatory and infected lesions such as infected lesions of the skin and/or mucosal membranes, surgical wounds, ulcerative lesions and burns. The presence of the DNA may increase the viscosity of solutions at the inflammatory site and may slow down or prevent the resolution of the inflammation. It may also cause mechanical difficulties, thus for example, where the enclosed inflammatory site is a joint, or at a joint, its functioning may be compromised. Thus it may be harder to flex or manipulate. The subject may have an autoimmune disorder, such as one characterised by the production of an immune response against DNA and in particular antibodies against DNA. In a preferred embodiment the subject may have, or be at risk of developing, systemic lupus erythematosus (SLE).

In a particularly preferred embodiment of the invention the subject will suffer from, or be at risk of, a respiratory disorder and in particular one where the viscosity of mucus or other pulmonary secretions is elevated due to the presence of DNA. The subject may, for example, have acute or chronic bronchial pulmonary disease, such as infectious pneumonia, bronchitis or tracheobronchitis, bronchiectasis, cystic fibrosis, asthma, tuberculosis, and/or fungal infections. The subject may have a respiratory tract infection. The subject may have sinusitis, sinus congestion or viral infections which infect the respiratory system such as a cold or flu. In a particularly preferred embodiment of the invention the subject will have cystic fibrosis. The subject may have pneumonia.

The disorder to be treated will typically involve infiltration of inflammatory cells to the inflammatory site and in particular into the respiratory system. These cells may be macrophages, granulocytes, lymphocytes or other white blood cells. They may be T cells or B cells. The granulocytes may be eosinophils, basophils or neutrophils and in particular will be neutrophils.

The number of inflammatory cells present at the site, and in particular neutrophils, may typically be increased by from 1 to 10,000 fold, preferably, from 10 to 5,000 fold, more preferably from 20 to 1,000 fold, still more preferably by from 50

to 500 fold and even more preferably from 100 to 200 fold. The proportion and number of given white cell types may be determined by standard staining techniques well known in the art. Typically, many of the infiltrating inflammatory cells undergo necrosis or lysis to release genomic DNA into the mucus of the individual. Thus the number and/or proportion of necrotic cells may be elevated typically by from 10 to 5000 fold, more preferably by from 50 to 2000 fold, still more preferably from 100 to 1000 fold and most preferably from 200 to 600 fold. In particular the necrotic infiltrating cells primarily responsible for the release of genomic DNA into the mucus will be granulocytes and especially neutrophils.

The genomic DNA from the inflammatory cells may be predominately in the form of long strands of viscous genomic DNA. The necrosis of these cells may also be accompanied by the release of other proteins from the inflammatory cells such as inflammatory mediators and antibacterial proteins such as proteases like neutrophil elastase and/or cathepsins. As well as genomic DNA other polymers may also be present in the mucus further contributing to its viscosity. In many cases actin polymers may also be present further contributing to viscosity.

A contributing factor to the migration of inflammatory cells to the lungs may be the presence of pathogens, irritants, allergens or pollutants in the lung. For example, and in particular in cystic fibrosis, the subject may have infections such as *Pseudomonas*, *Pneumococcus*, *Staphylococcus*, *Burkholderia*, *Haemophilus* and/or *Aspergillus* infections and in particular may display infection with *Haemophilus influenzae*, *Staphylcoccus aureus*, *Pseudomonas aeruginosa*, and/or *Burkholderia cepacia*. These infections may be longstanding, occur periodically or be new. They may be acute or chronic. In some cases the pathogen will display antibiotic resistance due to prolonged exposure to antibiotics during treatment.

Subjects may also, or alternatively, have a history of exposure to pollutants such as tobacco smoke or allergens such as pollen and these may contribute to the influx of inflammatory cells into the lung. The presence of pathogen, allergens and/or pollutants may also promote the infiltrating cells to undergo necrosis rather than be disposed of via processes such as apoptosis where genomic DNA is not believed to be released into the lung in substantial amounts.

The subject may have a history of exposure to pollutants and/or chemicals and in particular to those in an inhaleable form. In particular, the subject may be a tobacco smoker or a former tobacco smoker. Typically, the subject may be, or have been, a heavy smoker, smoking from 10 to 100, preferably from 20 to 60, more preferably from 25 to 50 and even more preferably from 30 to 40 cigarettes per day. The subject may have done so for several years such as from 5 to 50, preferably from 10 to 40, more preferably from 15 to 30 and even more preferably from 20 to 25 years. The individual may have been, or be, a pipe or cigar smoker. The individual may have chewed tobacco or products containing tobacco. In some cases the individual may have been passively exposed to tobacco smoke rather than smoke tobacco themselves. Thus the subject may, for example, have been cumulatively exposed to passive tobacco smoke for long periods due to their work, leisure and/or home environments. The subject may use inhaled narcotic such as, for example, marijuana or other narcotics which are typically mixed with tobacco prior to smoking.

The subject may have additionally been, or alternatively be, exposed to other chemical or environmental pollutants. Thus the subject may work, or have worked, in an environment which exposes them to chemicals and/or pollutants. Thus, for example, the subject may be a factory worker or miner such a coal miner. The subject may work in the construction industry. The subject may have been exposed to high levels of pollution, such as exhaust emissions from vehicles or other engines. The subject may have been exposed to smog or sulphur dioxide containing emissions. The subject may have a genetic predisposition to developing a disorder and in particular a respiratory disorder.

In one of the preferred embodiments of the invention the subject will have chronic airflow limitation (CAL). In one embodiment of the invention the subject may have chronic airflow limitation (CAL). CAL is a disease state characterized by airflow limitation that is not fully reversible. The airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases. In particular, although not exclusively, CAL is associated with smoking.

For the purposes of the present invention CAL may be defined as a condition where there is a progressive decline in lung function, with the affected subject having an FEV_1 of less than 80% of that predicted for an individual of that age/race and/or height and/or who displays a FEV_1/FVC ratio of less than 70%. In an especially preferred embodiment, the subject to be treated with CAL will have an FEV_1 of less than 75% of that predicted. Typically the reduction of FEV_1 is only partially reversible. In particular the reduction in FEV_1 is only partially reversible by treatment with bronchodilators such as, for example, β2 adrenergic agonists and in particular salbutamol.

The FEV_1 for an individual with CAL may be from 10 to 80% of that predicted. Typically, the FEV_1 of the subject will be from 10 to 75% of the predicted value. Preferably the subject may have a FEV_1 of from 60 to 75% the predicted value, more preferably from 40 to 60% of predicted and even more preferably a value below 40% of that predicted. The subject with CAL may have an FEV_1 of less than 70%, preferably less than 60%, more preferably less than 50% and even more preferably less than 40% of that predicted. The FEV_1 value for the subject will normally be measured against predicted values and adjusted for age/sex/race and/or height. Predicted values may be those taken from Coates (*supra*). The expected value, which the value obtained for the subject with CAL may be compared to, may be the average expected value for smokers, or non-smokers, or both groups combined, preferably the expected value will be that for non-smokers not suffering from CAL (Coates, *supra*).

The reduction in FEV_1 in the subject with CAL will only be partially reversible and in particularly only be partially reversible on administration of a bronchodilator. Thus, for example, an increase in FEV_1 over the base-line value for the subject (i.e. that prior to administration of the bronchodilator) of more than 15%, preferably more than 20% and even more preferably over 25% will be regarded as reversibility. The increase may begin from 5 to 30, preferably from 10 to 25, more preferably over a period of from 15 to 20 minutes after the administration of the bronchodilator. Preferably the increases will begin from 15 minutes after the administration of the bronchodilator. The increases persist typically from 3 to 6

hours, preferably from 4 to 5 hours and more preferably 4 hours. Typically the bronchodilator used in assessing reversability will be a β_2 adrenergic agonist such as salbutamol, or ipratropium. In one embodiment of the invention the reduction in FEV_1 may be totally, or almost totally refractory to treatment with bronchodilators.

The subject with CAL may also show similar minimal increases in FEV_1 with steroid drugs such as Becotide™ or Prednisolone™ although typically response to such agents will not be used to define reversibility. The increases will also occur over a longer time period such as after 2 to 3 days and, if the steroid drugs are continually administered, persist. If steroids are stopped the improvement may persist for from 12 to 48 hours, or for days, weeks or even months, such as from six hours to six weeks, preferably from 1 day to 3 weeks.

Tests to assess reversibility of reduction of FEV_1 will typically be performed when the subject is clinically stable and free from infection. The subject should not have taken, or have had administered to them, inhaled short-acting bronchodilators in the previous six hours, long-acting β agonists in the previous 12 hours or sustained release theophyllines in the preceding 24 hours.

In the diagnosis of CAL spirometric values should typically be measured before and after an adequate dose of inhaled bronchodilator is given to the subject. The dose should preferably be selected to be high on the dose/response curve and usually will be given by nebuliser to be certain it has been inhaled. A similar dose may be given with multiple inhalations from a metered dose inhaler and large volume spacer, but this is less preferred. A typical dosage/measurements protocol for a human subject would be:

- before and 15 minutes after 2.5 to 5mg nebulised salbutamol or 5 to 10 mg terbutaline;
- before and 30 minutes after 500 µg nebulised ipratropium bromide; or
- before and 30 minutes after both in combination.

Equivalent protocols may be used for non-human subjects.

The FVC of the subject may also be measured in the diagnosis of CAL. The ratio of FEV_1 to FVC can be used in the diagnosis of CAL. Subjects to be treated who have CAL will typically have an FEV_1/FVC value of less than 70%. The ratio of

FEV_1/FVC may be below 65%, preferably below 60%, more preferably below 55% and even more preferably below 55%. In an especially preferred embodiment, a subject will have a FEV_1/FVC of below 70% and also have a FEV_1 value of 80% or less of that predicted.

FVC (forced vital capacity) corresponds to the maximal volume of air forcibly exhaled from the point of maximal inhalation and can be measured using standard spirometry. In particular, the above specified values for FEV_1/FVC will be those after administration of a bronchodilator as outlined above. The reduction in FEV_1/FVC will typically show the same lack of reversibility as FEV_1 in subjects with CAL.

Spirometric assessment is the most preferred method for diagnosing CAL and hence method for identification of subjects who may be treated. Accordingly, in an preferred embodiment of the invention spirometric assessment will be used in the diagnosis of a subject who has CAL and hence is treatable using the invention. In addition, the symptoms displayed by the subject may also be assessed to help confirm a diagnosis of CAL. Typically diagnosis will involve spirometric assessment in combination with assessment of the symptoms of a subject as well as elucidating whether the subject has a history of exposure to risk factors. In some situations spirometric assessment may not be possible, particularly in situations where resources are limited, and CAL will be diagnosed by alternative means such as by looking for the symptoms of CAL listed herein and a history of exposure to risk factors for CAL. Although chest X-rays are not typically indicative of whether or not a subject has CAL, they may be used to diagnose other respiratory disorders, such as TB, and hence rule out CAL.

The subject with CAL will show, or have previously shown, an accelerated rate of decline of lung function compared to the average expected for an equivalent individual not suffering from CAL and in particular for an equivalent non-smoking individual. The subject may display shortness of breath and in particular may do so after physical exertion such as on exercise. Typically, this will not be induced by exposure to an allergen. The subject may also show increased incidence of bacterial or viral infection and this may exacerbate the condition.

The individual with CAL may show a rate of decrease in FEV_1 one, two, three, four or more times greater than the average annual value expected for an equivalent individual not suffering from CAL. For instance a subject over thirty may show an annual reduction of from 50 to 100, preferably from 50 to 80 and more preferably from 60 to 70 ml FEV_1/yr compared to a reduction of from 10 to 40 and typically of 20 to 40 ml of FEV_1/yr in the equivalent non-smoker. These values may also apply to non smoking sufferers of CAL such as where the disorder is caused by pollutants.

Subjects with CAL may display one or more, and sometimes all, of cough, increased sputum production, dyspnea, and/or a history of exposure to risk factors for the disease. In the case of cough, increased sputum and dyspnea these may have been present for extended periods of time such as at least a month, preferably six months, more preferably at least a year and still more preferably for at least two years. Chronic cough and sputum production often precede the development of CAL and may be indicative of individuals for which the invention can be used prophylactically to prevent the development of CAL.

Subjects with CAL may have a history of exposure to pollutants, including any of those mentioned herein and at any of the levels specified herein. In particular, the subject with CAL will have a history of exposure to tobacco and/or industrial pollutants.

The subject with CAL will typically be a mature adult. For example, the subject may be from 21 to 85, preferably from 25 to 70, more preferably from 30 to 60 and even more preferably from 40 to 50 years of age. The onset of any of, or a particular, symptom mentioned herein in association with CAL, will typically have been in adulthood. For example, the subject may have been at least 20, more preferably at least 25, still more preferably at least 30 and even more preferably at least 35 years of age before they experienced a particular symptom. In particular, the symptoms associated with more advanced stages of CAL, such as any of those mentioned herein, may have their onset at such later stages of life. Subjects with a genetic predisposition to developing CAL, such as those with α_1-antitrypsin deficiency, may develop CAL earlier. For example, they may display one or more, or

a particular, symptom at from 10 to 21, preferably from 12 to 18, or more preferably from 14 to 16 years of age. Alternatively, they may first show the symptom at any of the age ranges mentioned herein. The subject may have been diagnosed at any of the ages, or within any of the age ranges, specified herein. These ranges may also apply to any disorder which the subject may have and in particular those mentioned herein.

The subject may have a genetic predisposition to developing CAL and may display a family history of the condition. For example, the subject may have α_1-antitrypsin deficiency and hence be predisposed to developing CAL. Subjects at risk of developing CAL may have had low birth weights and/or a history of exposure to pollutants in the womb or in early life. The mother of the subject may be a smoker and may have continued to smoke during pregnancy. The subject may have had a history of severe childhood respiratory infection. Reduced maximal attained lung function, as measured by spirometry, may identify individuals who are at increased risk of developing CAL.

The subject may have an early stage of CAL in which the symptoms are generally moderate and may have periods of normality or of at least reduced symptoms. Alternatively, the subject may have a more developed stage of CAL in which the symptoms, and particular the reduction in FEV_1 is more pronounced. Preferably, the methods and medicaments of the invention are used, or administered at, an early stage of CAL so that they can arrest, slow or regress the increased rate in FEV_1 decline at as early a stage as possible.

CAL is typically a progressive disorder with the severity of CAL and the extent to which it has an impact on the sufferer increasing over time. Thus there may be a progressive manifestation in the symptoms of the disorder. Chronic cough is usually the first symptom to develop. It may initially be intermittent, but later may be present every day. The cough will typically be present throughout the day, rather than just at night and in the morning. In some cases, significant airflow limitation may develop without the presence of a cough. Small amounts of tenacious sputum are commonly raised after coughing bouts.

As CAL progresses the subject may experience dyspnea. The onset of dyspnea will often be one of the reasons why a human subject will first consult a

physician as it can be dehabilitating and also induce anxiety. As the lung function of the subject deteriorates further, breathlessness becomes more intrusive. The subject may display wheezing and chest tightness. The dyspnea may become worse during or after exercise. Respiratory infections may also exacerbate the condition and cause increased dyspnea. Human subjects may indicate that the dyspnea progressively impairs their ability to carry out physical labour and to exert themselves.

CAL sufferers are sometimes split into categories which reflect the stage and severity of the disease. This can help define the needs of a particular subject and what course of treatment should be administered. In one classification (GOLD Executive Summary, supra) subjects are split into the following categories:

Category 0 – At risk

Lung function, as measured by spirometry, is normal.

Chronic symptoms (cough, sputum, production).

Typically, human subjects will be unaware of abnormal lung function.

Category I: Mild CAL

Mild airflow limitation.

$FEV_1/FVC < 70\%$.

FEV_1 greater than, or equal to, 80% of that predicted.

With or without chronic symptoms (cough, sputum, production).

Human subjects may be unaware that their lung function is abnormal.

Category II: Moderate CAL

Worsening airflow limitation.

$FEV_1/FVC < 70\%$.

FEV_1 from 30 to 80% of that predicted (category can be subdivided into: IIA - FEV_1 from 50 to 80% of that predicted; and IIB - FEV_1 from 30 to 50% of that predicted).

With, or without, chronic symptoms (cough, sputum, production, dyspnea). The symptoms may typically become more pronounced on exertion. Human subjects are likely to be aware of the condition and have sort medical advice.

and the onset of symptoms will occur progressively over time. In CF the decline in lung function will have a more immediate onset, earlier on in life. A subject who has CAL will often be in their thirties, forties or even fifties before they become aware that they are suffering from the disorder. The major exception to the later onset of CAL, is in those sufferers who have α_1 anti-trypsin deficiency. Such subjects will display an earlier onset of CAL, such as in their teens or twenties when CAL is diagnosed, as they will be born with a genetic condition predisposing them to CAL. Subjects who have α_1 antitrypsin deficiency and CF can readily be distinguished from each other by biochemical and genetic tests to identify the nature of the disorder.

The subject to be treated will be a vertebrate animal and preferably will be a mammal. Typically the subject will be human. However, the invention also encompasses the treatment of animals with conditions which are the same as, or equivalent to, any of the human conditions mentioned herein. Thus the animal condition may have either an underlying pathology similar to, or the same as, that of the human condition. The animal may have one or more similar symptoms or characteristics of the human condition and in particular one or more of those listed above. Preferably, the animal will suffer from a condition which falls within the definition of any of these conditions listed above.

In cases where the subject is not human it may be a domestic animal or an agriculturally important animal. The animal may, for example, be a sheep, pig, cow, bull, poultry bird or other commercially farmed animal. In particular the animal may be a cow or bull and preferably is a dairy cow. The animal may be a domestic pet such as a dog, cat, bird, or rodent. In a preferred embodiment the animal may be a cat or other feline animal. The animal may be a monkey such as a non-human primate. For example, the primate may be a chimpanzee, gorilla, or orangutan. In a preferred embodiment of the invention the animal may be a horse and, for example, may be a racehorse. The animal may be a sports animal such as, for example, a greyhound.

Subject assessment

The present invention provides methods, medicaments, and preparations for

the hydrolysis of DNA in conditions caused by, or characterized by, the presence of extracellular endogenous DNA. One of the preferred ways of assessing the efficacy of various embodiments of the invention is in determining the amount of DNA present following treatment. Preferably, the change in the amount of DNA and in the range of DNA fragment sizes will typically be determined following administration of the medicaments of the invention compared to the situation prior to their administration. Any of the methods for measuring the amount, and fragment size, of DNA discussed herein may be utilised. The range of DNA fragment sizes may be determined by gel electrophoresis or other separation techniques based on separation of DNA by size.

The viscosity of the solution the DNA is present in, and in particular mucus viscosity, may be determined. Again, preferably the situation is compared prior to and post treatment. Any of the techniques discussed herein for measuring viscosity may be used such as, for example, techniques like compaction assays and assays using contortion pendulums or any other suitable rheological methods.

Preferably, the medicaments and methods of the invention will reduce the viscosity of the DNA containing solution by from 0 to 100%, preferably from 20 to 100%, more preferably from 50 to 100% and even more preferably from 75 to 100% of that prior to administration of the medicament. Preferably, the average length of DNA fragment present or the predominate fragment size will be reduced by from 1 to 1000 fold, more preferably by from 5 to 500 fold, more preferably by from 10 to 100 fold and still more preferably by from 20 to 50 fold. The average DNA fragment size, or the predominate fragment size, may typically be from 50 bp to 10 kb, preferably from 100 bp to 2,500 bp, more preferably from 250 bp to 1,000 bp and even more preferably from 400 bp to 600 bp following treatment. The treatment may result in a visible shift towards lower molecular weight DNA fragments as visualised, for example, by gel electrophoresis and ethidium bromide staining and in particular may reduce the proportion of DNA species on the gel above 2 kb, preferably above 5 kb, more preferably above 10 kb and still more preferably those above 20kb on the gel.

The above and below mentioned changes may be observed within hours or days of administration, such as from one hour to seven days, preferably from two

hours to two days and more preferably from four hours to 24 hours after administration.

The assays to assess viscosity and DNA may also be performed on samples taken from the subject to assess the optimal DNase and glycosaminoglycan concentrations and various other factors to use. Thus by performing experiments *in vitro* on samples such as sputum to determine how to optimally hydrolyse DNA, the medicament to be administered to the subject may be tailored to their needs. It may be possible to do this so that at times of particular severity of the disorder or particular circumstances the treatment regimen can be adjusted appropriately.

The administration of the medicaments may typically produce an improvement in the status of the subject. For example, in the case of respiratory disorders administration may result in a reduction of airway obstruction, mucus plugging, foaming and/or bubbling. The subject may show an increase in FEV_1 returning it to a value closer to that predicted for an equivalent subject not suffering from respiratory disorder.

In some cases, on treatment the subject will display an improvement of FEV_1 so that FEV_1 increases by from 25 to 100%, preferably by from 40 to 100%, more preferably from 60 to 100% and even more preferably from 80 to 100% of the predicted value. The improvement may also be smaller, such as from 5 to 10%, preferably from 15 to 25%, or more preferably from 20 to 25%. This may be accompanied by the subject experiencing increased ease in breathing, promoted expiration of mucus and reduced coughing. The subject may show increased ability to perform exercise and physically exert themselves.

The mucolytic effect of the DNase may also decrease the incidence of respiratory infections, in particular where the subject has cystic fibrosis or pneumonia. Thus the subject may show a decrease in infections with pathogens such as, for example *Pseudomonas*, *Pneumococcus*, *Staphylococcus*, *Burkholderia*, *Haemophilus* and/or *Aspergillus*. In particular, the patient may show reduced incidence of infection with *Haemophilus influenza*, *Staphylococcus aureus*, *Pseudomonas aeuroginosa* and/or *Burkholderia cepacia*. Subjects where such infections were chronic may no longer be infected or have decreased load of

pathogen following treatment. Pathogenic load and the nature of pathogens can be assessed using various microbiological techniques well known in the art such as staining, culturing and plating

In cases where the subject is suffering from an autoimmune disease characterised by the presence of antibodies reactive against DNA the efficacy of the invention may be assessed by monitoring for a decrease in the immune response against DNA in the subject. Thus typically the titre of antibodies against DNA present in the blood will be measured as may be the affinity of the antibodies present. The titre of the antibodies or their affinity may be decreased, for example, by a factor of two, four, five, ten, twenty, fifty or more fold. It may be decreased by more than 20%, preferably more than 40%, more preferably more than 60% and even more preferably by more than 90%. Assessment of antibody titre and affinity may be done using standard techniques such as ELISA assays. The severity of the disorder in the patient may be monitored.

The medicaments and methods of the invention will typically prevent or treat the disorder in question. They may ameliorate its severity and/or eliminate or reduce symptoms associated with the disorder. In particular they will reduce or eliminate characteristics of the disorder associated with the presence of extracellular DNA. Thus in respiratory disorders such as cystic fibrosis they will have a mucolytic effect reducing mucus viscosity. This will help in the clearance of the buildup of mucus and there may be increased expectoration of mucus. There may be an improvement in lung function and a decreased incidence, or severity, of infection. The subject may have increased sense of well being. In some cases on treatment the subject will display an improvement of FEV_1 so that FEV_1 is from 25 to 100%, preferably from 40 to 100%, more preferably from 60 to 100% and even more preferably from 80 to 100% of the predicted value.

FEV_1 is defined as the maximal forced volume which can be expired in one second starting from maximum inspiration (European Resp. Journal, 1993; 6: Suppl. 16 and Coates, Lung Function,: Assessment and Applications In Medicine, 4th Edition, Oxford, Blackwell, 1969). It can be measured by standard techniques well known in the art i.e. by spirometry.

The medicaments may increase life expectancy such as by, for example, 1 to 2 years, preferably from 2 to 4 years and even more preferably by more than 4 years.

Products

The present invention also provides a product comprising a glycosaminoglycan or a salt thereof which has an average molecular weight of from 8 to 40 kd and a DNAse. These may typically be in a solid or liquid form. It may be in the form of a powder. It may be lyophilised or frozen. It may also comprise other components such as water or a buffer. The product may take the form of an aqueous solution comprising both the DNase and the glycosaminoglycan or salt. The product may also comprise stabilising agents or preservatives. It may comprise agents which allow the product to be frozen without losing enzyme activity such as glycerol. The products may be formed, for example by mixing powders of glycosaminoglycan and DNase.

Administration and pharmaceutical compositions

The glycosaminoglycan or physiologically acceptable salt may be administered simultaneously, separately or sequentially to the DNase. Thus the two may be administered in the same medicament or as two separate medicaments and may be given at the same time, one after the other or separately. In embodiments where the two are to be administered separately, preferably the glycosaminoglycan, or salt will be administered prior to, or at the same time, as the DNase.

The glycosaminoglycan and DNase may typically be administered from one week to one minute apart, preferably from two days to one hour apart, even more preferably from 24 hours to 2 hours apart and still more preferably from 12 to 4 hours apart. The two may be administered from one minute to one hour apart, preferably from 10 minutes to 30 minutes apart and even more preferably from 15 to 25 minutes apart. The two may be administered from one minute to ten minutes apart, preferably from two minutes to eight minutes apart and more preferably from four to six minutes apart. In some cases they may be administered in succession, one directly after the other.

The two may be administered by the same route or via different routes,

preferably the two will be administered by the same route either as separate medicaments or in the same medicament. The glycosaminoglycan or salt and the DNase may be provided in two separate compositions, but may, for example, be mixed prior to addition to a delivery device or in the delivery device. The delivery device may have means to regulate the amount of each delivered to the subject. It may have means to mix the two or deliver each separately.

The medicaments and compositions of the present invention may be prepared by formulating the active agents, i.e the glycosaminoglycan or salt and/or the DNase, with a standard pharmaceutically acceptable carrier and/or excipient as is routine in the pharmaceutical art. The exact nature of the formulation will depend upon several factors including the particular glycosaminoglycan, salt or DNase employed and the desired route of administration. Suitable types of formulation are fully described in Remington's Pharmaceutical Sciences, 19th Edition, Mack Publishing Company, Eastern Pennsylvania, USA, the disclosure of which is included herein of its entirety by way of reference.

The necessary dose to be administered will normally be determined by a physician, but for the glycosaminoglycan, or salt will, for example, be from 0.01mg to 5g, preferably from 0.1 mg to 2.5g, more preferably from 1 mg to 1g, even more preferably from 10 mg to 500mg , still more preferably from 50 mg to 250 mg and even more preferably from 100 mg to 200 mg. These doses will typically be given once, twice or three times a day and will preferably be given once or twice a day and more preferably will be given twice a day.

In embodiments of the invention where the glycosaminoglycan is heparin, a derivative thereof or a physiologically acceptable salt of either thereof the dosage administered will typically be in the range of from 10 to 10,000 units per/kg body weight, preferably 100 to 10,000 units per/kg body weight, more preferably from 200 to 5000 units/kg body weight, even more preferably from 500 to 2000 units per/kg, and still more preferably from 1,000 to 1,500 units per/kg.

Typically the dose of DNase will be such that the concentration of DNase achieved at the target site is from 100 to 0.001 μg/ml, preferably from 50 to 0.1 μg/ml, still more preferably be from 25 to 0.5μg/ml, yet more preferably be from 10

to 1 μg/ml. The concentration achieved at the target site may be from 8 to 2 μg/ml and preferably from 4 to 2 μg/ml. These doses will typically be given once, twice or three times a day and will preferably be given twice a day.

The length of treatment may typically be from a day, one week, two weeks, a month, six months a year or more. In many cases the subject will remain on the medicaments of the invention permanently or for extended periods. This may in particular be the case where the subject has a genetic defect, such as where the subject has cystic fibrosis, and hence permanently has the condition. This may also be the case where one of the causative factors in the disorder is something the subject is continuously exposed to and cannot avoid, or does not wish to minimise exposure to. The subject may be a tobacco smoker, but not give up smoking tobacco.

In cases where the disorder is more short lived, or occurs periodically, the length of administration may be shorter and may be linked to the duration of the disorder. Thus, for example, where the condition is an abscess or other inflammatory situation such as one involving an enclosed inflammatory site, once the inflammation has been resolved the administration of the medicament may be ceased or reduced. The administration schedule may be coordinated so that higher levels of the medicaments are administered, or initiation of administration begins, at times when the disorder has increased severity. Thus in respiratory disorders such as, for example, cystic fibrosis or pneumonia this may be when the subject has an increase in airway obstruction, infection and/or inflammation. The medicament may also be given prior to exercise or other physical exertion.

In cases where the disorder involves a specific inflammatory site the medicament may be administered directly to the inflammatory site or in the region of the site. Thus the medicaments may, for example, be injected into the site, applied as a cream or on the arm *etc*. The medicament may be administered via an implant. Thus the invention also provides an implant comprising a medicament of the invention. In auto-immune disorders the medicaments may typically be systemically administered such as by intravenous injection. In particular this may be the case for SLE.

Preferably the medicaments of the invention may be administered via

inhalation and/or intra-nasally. Thus they may be delivered via the nose and/or mouth. Suitable methods for formulating and preparing medicaments to be administered via inhalation, installation and intranasally are well known in the art and may be employed in the present invention. The medicaments may also be administered via installation. In a preferred embodiment, the medicaments of the invention are suitable for administration by inhalation. For inhalation therapy the medicament may be in a solution useful for administration by liquid aerosol, metered dose inhalers, or in a form suitable for a dry powder inhaler. The medicament may be present in a blister pack or breakable capsule.

In some cases the medicaments may be administered via installation. In such cases, typically the medicament will be in liquid form and will be administered via an artificial airway such as, for example, an endotracheal tube. The liquid will typically be drawn up into a syringe and then expelled through the artificial airway into the respiratory tract of the subject. Installation is often used in an emergency context. In many cases it may be used where the subject has a relatively advanced form of CAL and has been admitted to hospital. Typically volumes such as from 1 to 20 ml, preferably from 2 to 10 ml, and even more preferably from 3 to 6 ml will be installed. Any method for delivery into the pulmonary tract may be used to deliver the medicaments to the invention.

In cases where the medicament is to be administered via the nose it may be, for example, in the form of nasal spray. The spray may be administered, for example, using an atomizer or nebulizer. In some cases the medicament may be in the form of nasal drop. These may be administered using a via a medicine dropper. For further discussion on nasal dosage forms see Remington's Pharmaceutical Sciences (*Supra*). Medicaments administered via the nose may include pH adjusters, emulsifiers or dispersing agents, preservatives, surfactants, gelling agents, or buffering agents as may the other medicaments of the invention. Most preferably the nasal dosage form will be isotonic with nasal secretions.

In some preferred embodiments, the medicaments of the present invention may be formulated as aerosols. The formulation of pharmaceutical aerosols is routine to those skilled in the art, see for example, Sciarra, J. in Remington's Pharmaceutical

Sciences (*supra*). The agents may be formulated as solution aerosols, dispersion or suspension aerosols of dry powders, emulsions or semisolid preparations. The aerosol may be delivered using any propellant system known to those skilled in the art. The aerosols may be applied to the upper respiratory tract, for example by nasal inhalation, or to the lower respiratory tract or to both. The glycosaminoglycan, salt or DNase may delivered using liposomes and nano-particle delivery methods. Liposomes, particularly cationic liposomes, may be used in carrier formulations.

Medicaments for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. In particular they may include a pharmaceutically acceptable excipient. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration. Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences (*supra*).

The medicaments and compositions of the invention may, for example, be buffered in such a way, if they include a DNase, that they will have a pH similar or identical to the pH optimum of the particular DNase being employed. For example, the pH employed may be within 2, preferably 1, more preferably 0.5, even more preferably 0.2 and still more preferably 0.1 pH units of the pH optimum of the enzyme being employed. In some cases, a DNAse may be active over a wide range of pH and the pH chosen will be that closest to physiological pH, or at least the enzyme will be active at least partially at physiological pH and hence may be buffered at physiological pH or at least a pH within 2, preferably 1, more preferably 0.5, even more preferably 0.2 and still more preferably 0.1 pH units of optimal pH.

The medicaments may include various constituents to optimise their suitability for the particular delivery route chosen. The viscosity of the medicaments may be maintained at a desired level using a pharmaceutically acceptable thickening agent. Thickening agents that can be used include methyl cellulose, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, carbomer, polyvinyl alcohol, alginates, acacia, chitosans and combinations thereof. The concentration of the

thickening agent will depend upon the agent selected and the viscosity desired.

In some embodiments, and in particular where intranasal delivery is to be used, the medicaments may comprise a humectant. This may help reduce or prevent drying of the mucus membrane and to prevent irritation of the membranes. Suitable humectants include sorbitol, mineral oil, vegetable oil and glycerol; soothing agents; membrane conditioners; sweeteners; and combinations thereof.

The medicaments may comprise a surfactant. Suitable surfactants include non-ionic, anionic and cationic surfactants. Examples of surfactants that may be used include, for example, polyoxyethylene derivatives of fatty acid partial esters of sorbitol anhydrides, such as for example, Tween 80, Polyoxyl 40 Stearate, Polyoxy ethylene 50 Stearate, fusieates, bile salts and Octoxynol.

The medicaments of the present invention may be delivered by any device adapted to introduce one or more therapeutic compositions into the upper and/or lower respiratory tract. In some preferred embodiments, the devices of the present invention may be metered-dose inhalers. The devices may be adapted to deliver the therapeutic compositions of the invention in the form of a finely dispersed mist of liquid, foam or powder. The device may use a piezoelectric effect or ultrasonic vibration to dislodge powder attached on a surface such as a tape in order to generate mist suitable for inhalation. The devices may use any propellant system known to those in the art including, but not limited to, pumps, liquefied-gas, compressed gas and the like.

Devices of the present invention typically comprise a container with one or more valves through which the flow of the therapeutic composition travels and an actuator for controlling the flow. Suitable devices for use in the present invention may be seen, for example, in Remington's Pharmaceutical Sciences (*supra*). The devices suitable for administering the medicaments of the invention include inhalers and nebulisers such as those typically used to deliver steroids to asthmatics. In some cases, where the subject is for example a child, a spacer may be used to facilitate effective administration from the inhaler.

Various designs of inhalers are available commercially and may be employed to deliver the medicaments of the invention. These include the Accuhaler, Aerohaler,

Aerolizer, Airmax, Autohaler, Clickhaler, Diskhaler, Easi-breathe inhaler, Fisonair, Integra, Jet inhaler, Miat-haler, Novolizer inhaler, Pulvinal inhaler, Rotahaler, Spacehaler, Spinhaler, Syncroner inhaler and Turbohaler devices.

In cases where the glycosaminoglycan and/or DNAse are administered in the form of particles or droplets, the particle/droplet size and/or other properties of the particle/droplet may be chosen to ensure that the particles are delivered to a particular region of the respiratory tract. For example, they may be designed to reach only the upper or lower parts of the respiratory tract. In cases where the glycosaminoglycan, salt or therapeutic agent are delivered in an aqueous form preferably the solution will be isotonic to help ensure effective delivery to the subject.

In embodiments where it is desired to administer the medicaments to, or via, the respiratory tract the particle size of the medicament may be chosen on basis of the desired part of the respiratory tract which it is desired to administer the medicament to. In particular particles with a diameter of 10 μM are thought to be effective in reaching the lower parts of the respiratory tract and hence may be employed where such a site is the desired target for the medicaments. In embodiments, where it is desired to deliver the medicament to the lower parts of the respiratory tract, such as alveoli for example, the diameter of the particles administered may be less than 10 μM, preferably less than 8 μM, more preferably less than 6 μM and even more preferably less than 4 μM. In a preferred embodiment the particles may have a diameter of 3 μM or less and more preferably may have a diameter of 2μM or less. In an especially preferred embodiment the particles will have a diameter of from 3 to 5μM. In some cases the particles administered may be less than 1000 nm, preferably less than 500 nm, more preferably less than 250 nm and still more preferably less than 100 nm in diameter. The sizes may refer to particles of solid matter or droplets of solutions and suspensions.

The size of particles necessary to penetrate to a specific part of the respiratory tract will be known in the art and hence the particle size can be chosen to suit the target size. Techniques such as milling may be used to produce the very small particles necessary. In some cases the desired part of the respiratory tract may be the upper respiratory tract and hence larger particles sizes may be employed. The density

of the particles and their shape may also be chosen to facilitate their delivery to the desired site.

The medicaments of the invention may take a variety of forms. In case where they are to be administered via the respiratory tract they may be in the form of powders, powder microspheres, solutions, suspensions, gels, nano-particle suspensions, liposomes, emulsions or microemulsions. The liquids present may be water or other suitable solvents such as a CFC or HFA. In the case of solutions and suspensions these may be aqueous or involve solutions other than water.

The compositions of the invention may also be coated onto implants, devices or materials to be introduced into the body. In such embodiments they may be released from the surface of the object, preferably slowly.

The present invention also provides a pharmaceutical composition comprising:

a glycosaminoglycan or a physiologically acceptable salt thereof which has an average molecular weight of from 8 to 40 kd;

- a DNAse; and
- a pharmaceutically acceptable excipient.

The excipient may be any of those mention herein, or any of the carriers. The composition may take the form of any of the medicaments discussed herein or any of the characteristics or features specified herein for them. The DNase and glycosaminoglycan or salt may be any of those discussed herein.

Non-clinical applications

Although the principle use of the invention is in a clinical context, DNases are also used for a number of non-clinical applications. Thus the demonstration of the synergistic effect of glycosaminoglycan on DNase activity means that the amount of DNase necessary in such applications may be decreased or alternatively that a higher level of DNase activity may be achievable using the same amount of DNase.

Thus the present invention provides for the use of a glycosaminoglycan, or a physiologically acceptable salt thereof which has an average molecular weight of from 8 to 40kd to increase the activity of a DNase. In particular this will be of use *in vitro*.

The non-clinical uses of the invention include, for example, in laboratory experiments and/or research. For, example DNase is sometimes used to remove contaminating DNA from RNA preparations and in particular from mRNA prior to cDNA synthesis. It is also used to reduce the viscosity in microinjection chambers for embryo micro-injection and other systems where increases in viscosity due to the presence of DNA may cause problems. The invention may be used to increase DNase activity in situations where the enzyme is being used to reduce viscosity of a solution by cleaving DNA.

The products and methods of the invention may be used in any non-clinical application involving DNase. Additional purification steps may be introduced into procedures to remove the glycosaminoglycan, if so desired or necessary, subsequent to or following the DNase treatment. Any of the parameters described herein in relation to the clinical applications of the invention may be equally applicable to the non-clinical applications and *vice versa*.

Kits

The present invention also provides kits comprising: a glycosaminoglycan or a salt thereof, of average molecular weight of from 8 to 40kd; a DNase; and packaging. The glycosaminoglycan or salt and the DNase may be provided in the kits in any of the forms discussed herein. The kit may also comprise additional reagents or assays for performing the various assays and techniques which may be performed using DNase and in particular laboratory techniques using the enzyme. The kit may also additionally comprise instructions on how to perform the techniques.

The invention also provides kits intended for use in a therapeutic context which comprise:

- a glycosaminoglycan or a physiologically acceptable salt thereof, of average molecular weight of from 8 to 40kd;
- a DNase;
- instructions for the sequential, simultaneous or separate administration of the glycosaminoglycan, or salt and the DNase to a patient suffering from a disorder characterised by the presence of endogenous extracellular DNA in the

subject to be treated; and

- packaging.

The instructions may take the form of a label on the packing. The kit may additionally comprise means for administering the glycosaminoglycan or salt and/or the DNase such as any of those discussed herein.

The following Examples illustrate the invention.

Example 1: Demonstration of synergy of DNase and heparin *in vitro*

The effect of heparin on the activity of DNase *in vitro* was determined. Calf thymus DNA (5 μg/ml) was incubated with a range of DNase concentrations in the presence or absence of unfractionated heparin (160 USP units per mg of a heparin sodium salt from porcine intestinal mucosa – obtained from Calbiochem, catalogue number 375095) was used at a concentration of 1 mg/ml for 1 hour at 37°C. All solutions were prepared in sodium phosphate buffer containing 1.2mM magnesium sulphate. Following DNase treatment the amount of intact DNA was estimated using Hoechst stain and quantifying the associated fluorescence (Labarca & Paider, 1980, Anal. Biochem., 102:344-352). Bovine DNase I was used and had 2,500 kunitz units per mg (the same DNase was used in Examples 2 and 3). The results obtained are shown in Figure 1.

The presence of heparin significantly increased the activity of the DNase, resulting in a more rapid breakdown of the DNA. However, experiments using low molecular weight heparin of average molecular weight 6kd in the concentration range 0.01 μg/ml to 10 mg/ml had no effect on DNase activity (LMWH – a sodium heparin salt from porcine intestinal mucosa with MW6000 was employed – obtained from Sigma, catalogue number D7037). Thus, the results show that at concentrations of DNase typically achieved in the airways of cystic fibrosis patients receiving inhaled DNase therapy (~ 2.9 μg/ml) unfractionated heparin significantly enhanced the effect of DNase.

Example 2: Agarose gel electrophoresis determination of the effect of heparin on the breakdown of DNA by DNase

Agarose gel electrophoresis was used to assess the breakdown of DNA by DNAase and the effect of the addition of heparin. Calf thymus DNA (1mg/ml) was incubated with DNase (1 μg/ml) for a range of time periods at 37°C. Reactions were run containing DNA and DNase with either a 5, 15, 30, 45 or 50 minute incubation period. Duplicate reactions were performed in the presence and absence of 10 mg/ml unfractionated heparin. Control reactions with DNA alone were also run. All solutions were prepared in sodium phosphate buffer containing 5mM magnesium sulphate. Following incubation a sample of each solution was run on a 2 % agarose gel and stained for DNA using ethidium bromide. The results obtained are shown in Figure 2. These show that the hydrolysis of DNA to small fragments is enhanced in the presence of heparin, and that the effect was time-dependent.

Example 3: Atomic Force Microscopy (AFM) determination of the effect of heparin on the breakdown of DNA by DNase

The effect of heparin on the breakdown of DNA by DNase was assessed by atomic force microscopy. Atomic force microscopy is a powerful technique capable of imaging surfaces at nanometre or sub-nanometre resolution. Since the technique does not require specimens to be metal coated or stained, non-invasive imaging can be performed on surfaces in their native states.

The principle of AFM relies on the use of a small, sharp, square-pyramidal silicon nitride tip which is brought into close proximity to the surface where intermolecular forces acting between the tip and the surface cause the cantilever to bend. Images of the surface are obtained by recording the cantilever deflections, as detected by a laser beam focussed on the top of the cantilever, as the sample is scanned.

Calf thymus DNA (0.1 mg/ml) was incubated with DNase (2.9 μg/ml) in the absence and presence of a range of unfractionated heparin concentrations at 37°C for 1 hour. All solutions were prepared in 0.2 μm filtered, DNase and RNase free water containing 5mM magnesium sulphate. Following incubation, samples were dialysed by centrifugation for 10 mins at 20,000g in microcentrifuge dialysis tubes (5000 MW cut-off). The samples were resuspended in water and centrifugation repeated prior to

final resuspension. 10 µl of sample was added to freshly cleaved ruby muscovite mica and dried prior to AFM imaging. AFM was performed in air under ambient conditions using a TopoMetrix TMX2000 Scanning Probe Microscope (ThermoMicroscopes, Bicester, UK) with a 70'70'12 mm tripod piezoelectric scanner. Topography measurements, in contact mode, were made using a 'V'-shaped silicon nitride cantilever (length 200nm, nominal spring constant (K) 0.21 Nm-1; Part. No. 1530-00, ThermoMicroscopes, Santa Clara, CA, USA) bearing an integrated standard profile tip.

The results obtained are shown in Figure 3. These show the results for samples comprising: 0.1 mg/ml DNA (Panel A); 0.1 mg/ml DNA and 2.9 µg/ml DNase (Panel B); 0.1 mg/ml DNA, 2.9 µg/ml DNase and 1 µg/ml heparin (Panel C); and 0.1 mg/ml DNA, 2.9 µg/ml DNase and 10 µg/ml heparin (Panel D). The results show that untreated DNA forms a regular polymer network and in the presence of DNase the pore size of the network increases. Addition of heparin at low concentrations to DNase results in the depolymerisation of DNA and the destruction of the polymer networks.

Example 4: Determination of the effects of a variety of glycosaminoglycans on DNase activity using fluorescence staining

Following the demonstration of the ability of heparin to synergistically increase the activity of DNase, a variety of other glycosaminoglycans were assessed for their effect on DNase activity.

Calf thymus DNA (at a concentration of 0.1 mg/ml) was incubated with DNase (at a concentration of 2.9 µg/ml) in the presence of a range of concentrations of different glycosaminoglycans, namely heparin, chondroitin sulphate, heparan sulphate (a sodium salt of heparan sulphate from porcine intestinal mucosa was employed – obtained Sigma, catalogue number H-9902), low molecular weight heparin (LMWH). The chondroitin sulphate employed was a 70:30 mixture of sodium salts of chondroitins A and C obtained from bovine trachea (Sigma C-8529). Dextran sulphate was tested as a non-glycosaminoglycan control (a dextran sulphate sodium salt was used – obtained from Sigma, catalogue number D-7037). The

incubation period was for one hour at 37°C. Following the incubation Hoechst stain (5 μg/ml) was added and fluorescence measured (excitation 355 nm, emission 460 nm). All solutions were prepared in sodium acetate buffer containing 5 mM magnesium sulphate.

The results obained are shown in Figure 4. All of the glycosaminoglycans enhanced the activity of DNase apart from the low molecular weight heparin. Dextran sulphate, the non glycosaminoglycan control did not enhane activity. Chondroitin sulphate gave particularly good results.

Example 5: Determination of the effect of chondroitin sulphate on DNase efficacy on cystic fibrosis sputum

Given the increase in DNase activity in the presence of chondroitin sulphate the effect of this glycosaminoglycan on DNase activity on cystic fibrosis sputum was assessed. Cystic fibrosis is one of a number of disorders characterized by an elevation in sputum viscosity due to the presence of endogenous DNA. The assessment was done using a barrier assay.

Cystic fibrosis sputum was homogenised prior to the addition of DNase (2.9 μg/ml) and chondroitin sulphate (a 70:30 mixture of chondroitins A and C at a range of concentrations was used) on a 10 % v/w basis. Mixing was performed using a 19-gauge needle. Carboxylate-modified fluorescent beads were added to sputum sample in a 1:1 ratio (v/w). After vortexing, 20 μl of sample was added to each upper well of a micro-boyden chamber. The upper and lower wells of the chamber were separated by an 8 μm polycarbonate filter, and the lower wells contained PBS. The chamber was centrifuged briefly at 1000 rpm to remove air bubbles prior to incubation at 37°C, 900 rpm, for 4 hours in the dark. Following incubation the chamber was dismantled and the fluorescence of the solution in the lower wells measured.

The results obtained are shown in Figure 5. These show an increase in the level of transport of microspheres, compared to the level seen with DNase alone, when chondroitin sulphate. The increase was seen even at relatively low concentrations of chondoitin sulphate.

Example 6: Atomic force Microscopy (AFM) determination of the effect of chondroitin sulphate on DNA structure in the presence of DNase

AFM imaging was carried out as discussed in Example 3 above.

Calf thymus DNA (0.1 mg/ml) was incubated with DNase (2.9 μg/ml) ± chondroitin sulphate (a 70:30 mixture of chondroitins A and C) at a concentration of 0.01μg/ml 37°C for 1 hour. All solutions were prepared in 0.2 μm filtered, DNase and RNase free water containing 5 mM magnesium sulphate.

Following incubation, samples were centrifuged for 10 mins at 20,000g in microcentrifuge dialysis tubes. The sample was resuspended in water and centrifugation repeated prior to final resuspension. 10 μl of sample was added to freshly cleaved ruby muscovite mica and dried prior to AFM imaging. AFM was performed in air under ambient conditions using a TopoMetrix TMX2000 Scanning Probe Microscope (ThermoMicroscopes, Bicester, UK) with a 70 ' 70 ' 12 mm tripod piezoelectric scanner. Topography measurements, in contact mode, were made using a 'V'-shaped silicon nitride cantilever (length 200 nm, nominal spring constant (K) 0.21 Nm-1; Part. No. 1530-00, ThermoMicroscopes, Santa Clara, CA, USA) bearing an integrated standard profile tip.

The results obtained are shown in Figure 6. They show an increase in the breakdown of the DNA network in the presence of both chondroitin sulphate and DNase compared to that seen with DNase alone.

CLAIMS

1. Use of a glycosaminoglycan or a physiologically acceptable salt thereof, in the manufacture of a medicament for treating a subject with a disorder characterised by the presence of endogenous extracellular DNA wherein the subject is also being treated with a DNase and the glycosaminoglycan, or salt has an average molecular weight of from 8 to 40 kd

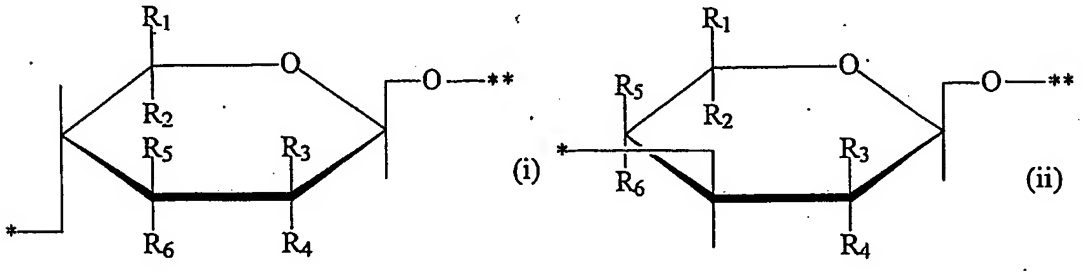
2. Use of a DNase in the manufacture of a medicament for treating a subject with a disorder characterised by the presence of endogenous extracellular DNA wherein the subject is also being treated with a glycosaminoglycan or a physiologically acceptable salt thereof, and the glycosaminoglycan, or salt has an average molecular weight of from 8 to 40kd.

3. Use according to claim 1 or 2, wherein the glycosaminoglycan or the physiologically acceptable salt comprises repeating disaccharide units of general formula (1)

-[A-B]- (1)

wherein :

each A is the same or different and represents a moiety of formula (i) or (ii)



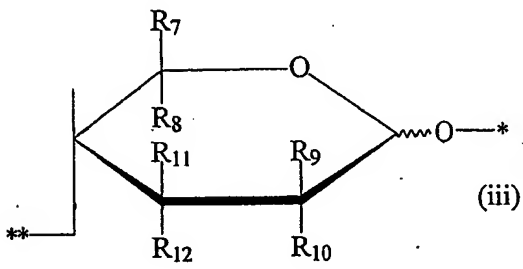
wherein:

- one of R_1 and R_2 is hydrogen, and the other is $-CO_2H$, $-SO_3H$ or $-CH_2OR$

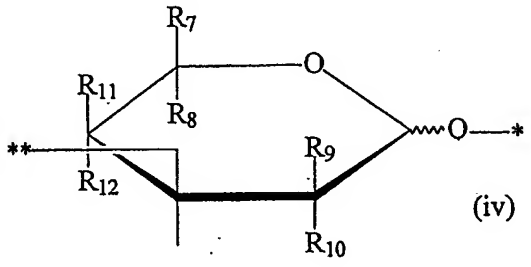
wherein R is hydrogen or $-SO_3H$;

- one of R_3 and R_4 is hydrogen, and the other is -OR wherein R is hydrogen or $-SO_3H$;
- one of R_5 and R_6 is hydrogen, and the other is -OH;
- * represents a direct bond to an adjacent hydrogen atom or B moiety; and
- ** represents a direct bond to an adjacent B moiety;

each B is the same or different and represents a moiety of formula (iii) or (iv)



(iii)



(iv)

wherein:

- one of R_7 and R_8 is hydrogen and the other is $-CH_2OH$ or $-CH_2OSO_3H$;
- one of R_9 and R_{10} is hydrogen and the other is -NHAc, $-NH_2$ or $-NHSO_3H$;
- one of R_{11} and R_{12} is hydrogen and the other is -OH or $-OSO_3H$;
- indicates a bond in either stereochemical orientation;
- * represents a direct bond to a hydrogen atom or an adjacent A moiety;
- ** represents a direct bond to an adjacent A moiety.

or a physiologically acceptable salt thereof.

4. Use according to any one of the preceding claims, wherein the glycosaminoglycan is a heparin or a derivative thereof.

5. Use according to any one of the preceding claims, wherein the sodium salt of heparin or heparin sulphate is used.

6. Use according to any one of claims 1 to 3, wherein the glycosaminoglycan is

chondroitin sulphate A, chondroitin sulphate C, chondroitin sulphate D, chondroitin sulphate E hyaluronic acid, keratan sulphate, heparan sulphate or a derivative of any thereof.

7. Use according to claims 6, wherein the glycosaminoglycan is chondroitin sulphate A, chondroitin sulphate C or heparan sulphate or a derivative of any thereof.

8. Use according to any one of the preceding claims wherein the DNase is a type I DNase.

9. Use according to claim 8, wherein the DNase is a human type I DNase.

10. Use according to any one of the preceding claims, wherein the DNase is a recombinant DNase.

11. Use according to any one of the preceding claims, wherein the glycosaminoglycan or salt has an average molecular weight of from 12 to 18 kd.

12. Use according to any one of the preceding claims, wherein the glycosaminoglycan or salt has anti-coagulant activity.

13. Use according to any one of the preceding claims, wherein the glycosaminoglycan, or salt has not been subjected to fragmentation and/or depolymerisation.

14. Use according to any one of the preceding claims, wherein the ratio of the amount of glycosaminoglycan or salt to the amount of DNase administered is from 500:1 to 1:500 unit for unit.

15. Use according to claim 14, wherein the ratio is from 25:1 to 1:25.

16. Use according to any one of the previous claims, wherein the glycosaminoglycan or salt and the DNAse are administered at the same time.

17. Use according to any one of the preceding claims, wherein the glycosaminoglycan or salt is administered before or after the DNase.

18. Use according to any one of the preceding claims, wherein the glycosaminoglycan or salt and the DNase are administered as separate medicaments.

19. Use according to any one of the preceding claims, wherein the disorder is a respiratory disorder characterised by the presence of endogenous extracellular DNA in the lung.

20. Use according to any one of the preceding claims, wherein the disorder is selected from cystic fibrosis, chronic airflow limitation and pneumonia.

21. Use according to any one of the preceding claims, wherein the subject has an FEV_1 of less than 75% of the expected value for an equivalent subject not having the disorder.

22. Use according to any one of claims 1 to 18, wherein the disorder is systemic lupus erythematosus (SLE).

23. Use according to any one of claims 1 to 18, wherein the disorder is a disorder characterised by the presence of endogenous extracellular at an enclosed inflammatory site.

24. Use according any one of the preceding claims, wherein the glycosaminoglycan, or salt and/or the DNase are administered via inhalation, intra-nasally and/or via installation.

25. A composition comprising a glycosaminoglycan or a salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40 kd and a DNAse.

26. A composition according to claim 25, which is a pharmaceutical composition comprising:

- a glycosaminoglycan or a physiologically acceptable salt thereof which has an average molecular weight of from 8 to 40 kd;
- a DNAse; and
- a pharmaceutically acceptable excipient, carrier or diluent.

27. Products comprising a glycosaminoglycan or a physiologically salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd, and a DNase for simultaneous, separate or sequential use in the treatment of a disorder characterised by the presence of endogenous extracellular DNA in the subject to be treated.

28. A method of improving the efficacy of the treatment with a DNase of a subject having a disorder characterised by the presence of endogenous extracellular DNA, which method comprises the steps of:

(a) administering to said subject a glycosaminoglycan or a physiologically acceptable salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd to the subject; and

(b) administering to said subject a DNase.

29. An agent for treating a subject having a disorder characterised by the presence of endogenous extracellular DNA, the agent comprising a glycosaminoglycan or a physiologically acceptable salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd and wherein the said subject is being treated with a DNase.

30. An agent for treating a subject having a disorder characterised by the presence of endogenous extracellular DNA, the agent comprising a DNase and wherein the said subject who is being treated with a glycosaminoglycan or a physiologically acceptable salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd.

31. A kit comprising:

- a glycosaminoglycan or a physiologically acceptable salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd;
- instructions for the sequential, simultaneous or separate administration of the glycosaminoglycan or salt to a subject suffering from a disorder characterised by the presence of endogenous extracellular DNA and being treated with a DNase; and
- packaging.

32. A kit comprising

- a DNase;
- instructions for the sequential, simultaneous or separate administration of the DNäse to a subject suffering from a disorder characterised by the presence of endogenous extracellular DNA and being treated with a glycosaminoglycan or a pysiologically acceptable salt thereof, having an average molecular weight of from 8 to 40kd; and
- packaging.

33. A composition according to claim 25 or 26, products according to claim 27, a method according to claim 28, an agent according to claim 29 or 30 or a kit according to claim 32 or 33, wherein:

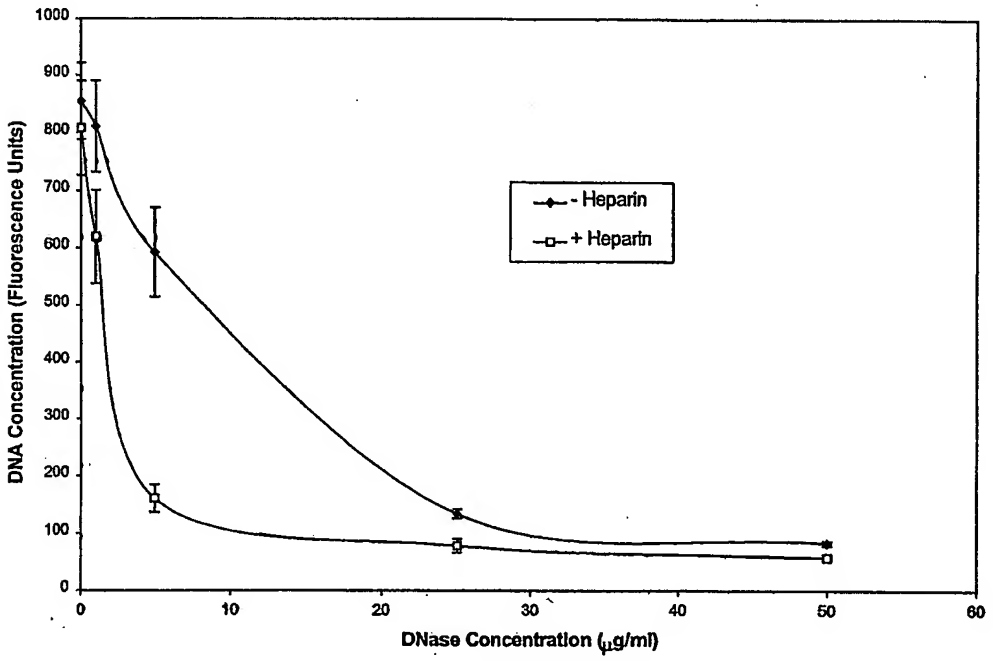
- the glycosaminoglycan or physiologically salt is as defined in any one of claims 3 to 7 and 11 to 13;
- the DNAse is as defined in any one of claims 8 to 10; and/or

- the ratio of glycosaminoglycan or salt to DNase is as defined in claim 14 or 15.

34. Products according to claim 27 or 33, a method according to claim 28 or 33, an agent according to claim 29, 30 or 33 wherein the disorder is as defined in any one of claims 19 to 33.

35. A nebuliser or other liquid aerosol device, dry powder inhaler or metered dose inhaler comprising a composition according to claim 26 or 33.

36. Use of a glycosaminoglycan or a physiologically acceptable salt thereof, the glycosaminoglycan or salt having an average molecular weight of from 8 to 40kd to increase the activity of a DNAse *in vitro*.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800
700
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500
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300
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0
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30
40
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60
DNase Concentration (μg/ml)
- Heparin
+ Heparin

Figure 1

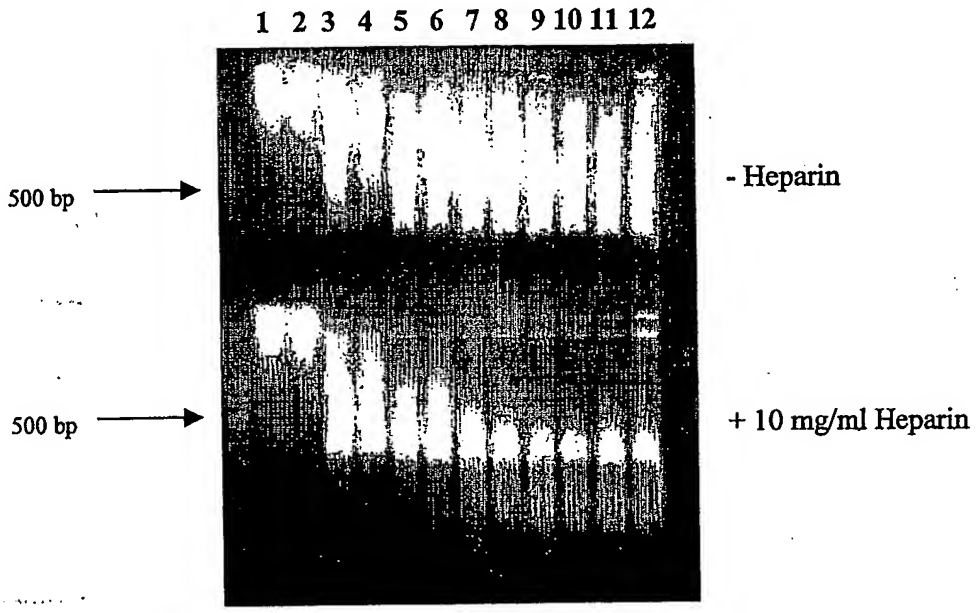
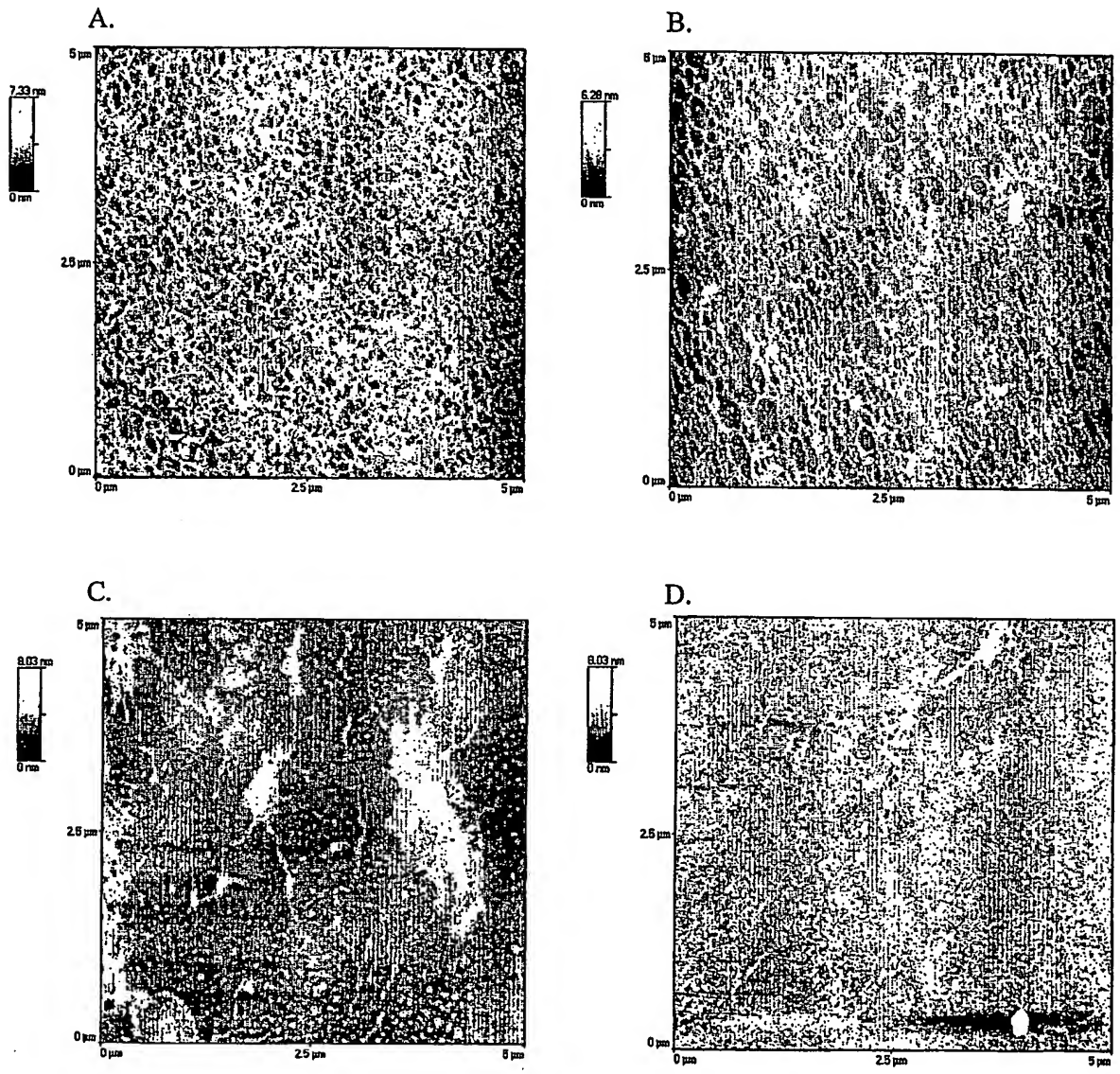


Figure 2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2.5 µm
0 µm
0 µm
2.5 µm
5 µm
B.
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0 nm
5 µm
2.5 µm
0 µm
0 µm
2.5 µm
5 µm
C.
8.03 nm
0 nm
5 µm
2.5 µm
0 µm
0 µm
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D.
8.03 nm
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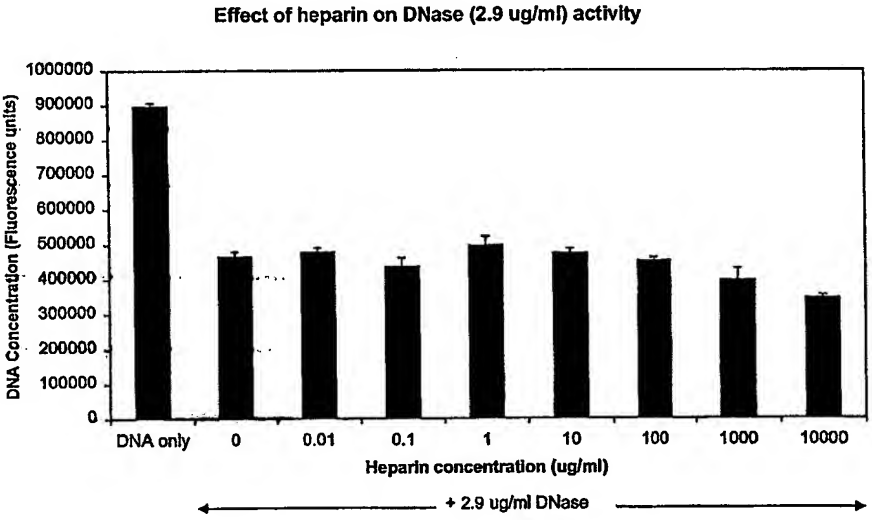
0 µm
0 µm
2.5 µm
5 µm

Figure 3

A.


Effect of heparin on DNase (2.9 ug/ml) activity
DNA Concentration (Fluorescence units)
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
DNA only
0
0.01
0.1
1
10
100
1000
10000
Heparin concentration (ug/ml)
+ 2.9 ug/ml DNase

B.

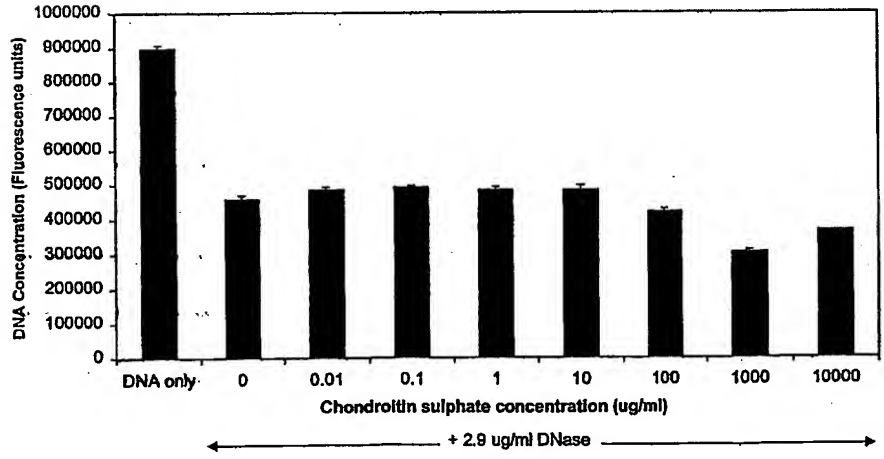
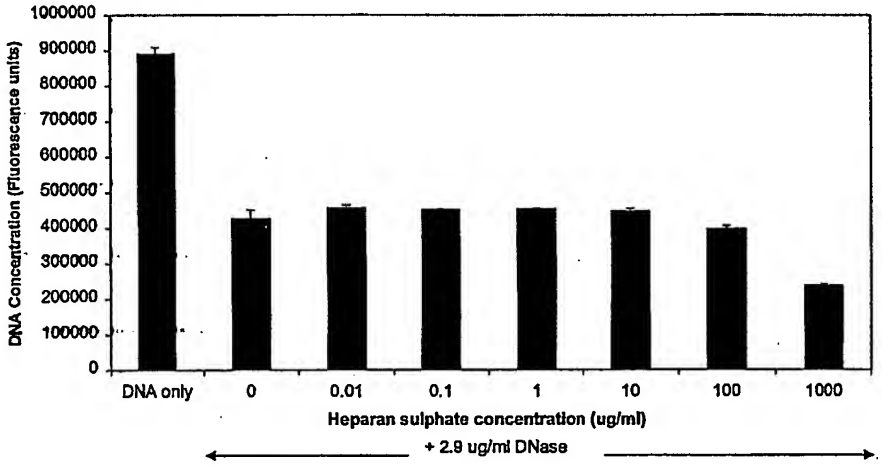

Effect of chondroitin sulphate on DNase (2.9 ug/ml) activity
DNA Concentration (Fluorescence units)
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
DNA only
0
0.01
0.1
1
10
100
1000
10000
Chondroitin sulphate concentration (ug/ml)
+ 2.9 ug/ml DNase

Figure 4

C.



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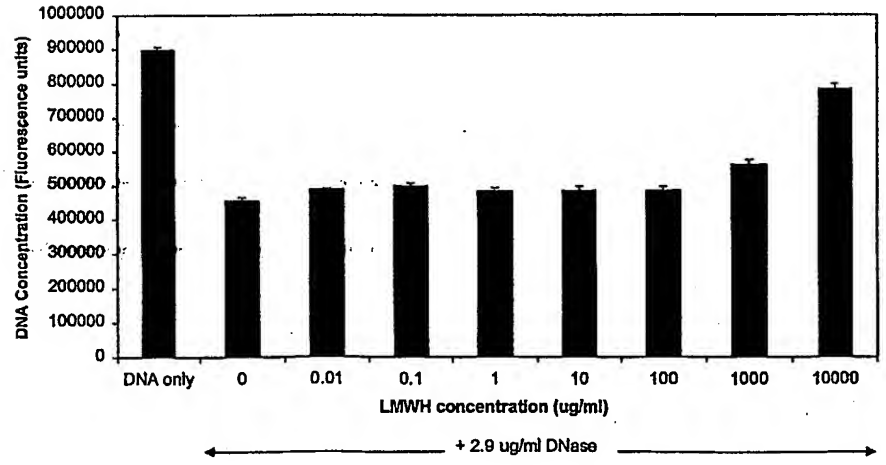


Figure 4 (continued)

E.

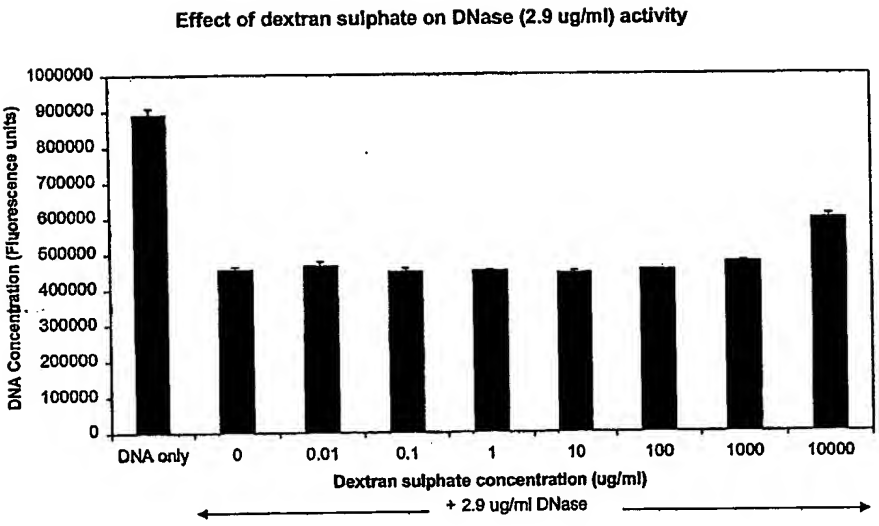


Figure 4
(continued)

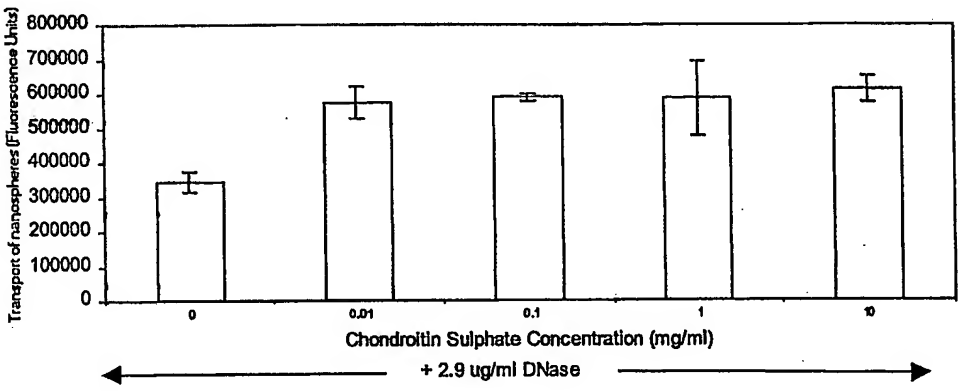
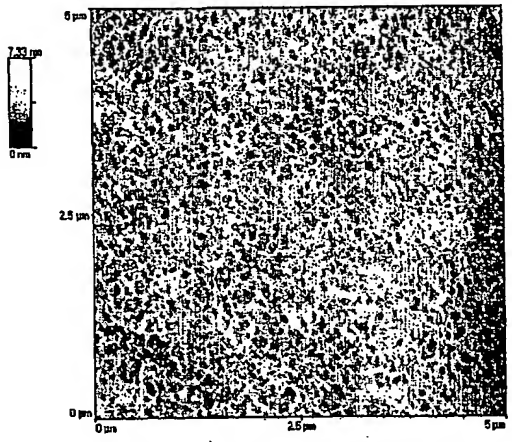
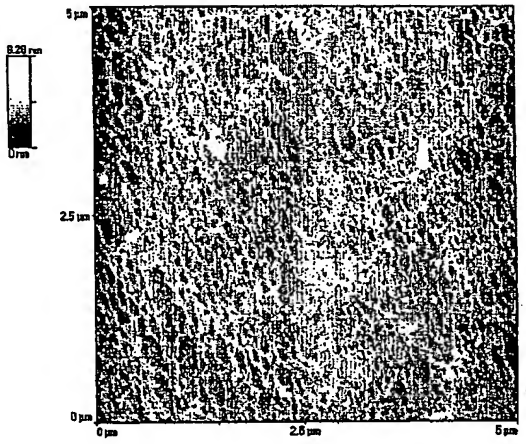

Transport of nanospheres (Fluorescence Units)
800000
700000
600000
500000
400000
300000
200000
100000
0
0
0.01
0.1
1
10
Chondroitin Sulphate Concentration (mg/ml)
+ 2.9 ug/ml DNase

Figure 5

A.



B.



C.

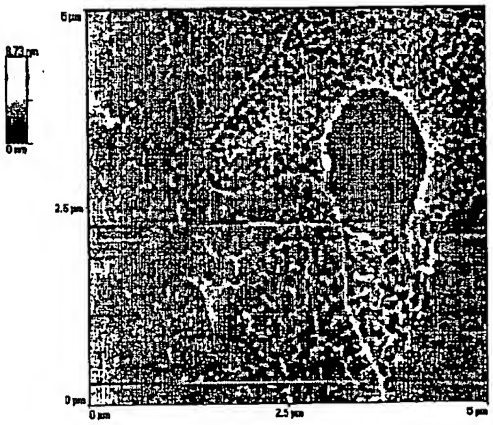


Figure 6

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Glu Glu Glu Ala Leu Asn Ile Ser Asp His Tyr Pro Val Glu Val Glu
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<400> 2

Ala Met His Tyr Pro Thr Ala Leu Leu Phe Leu Ile Leu Ala Asn Gly
1               5                   10                  15
Thr Gln Thr Phe Arg Ile Cys Ala Phe Asn Ala Gln Arg Leu Thr Leu
            20                  25                  30
Pro Lys Val Ala Arg Glu Gln Val Met Asp Thr Leu Val Arg Ile Leu
        35                  40                  45
Ala Arg Cys Asp Ile Met Val Leu Gln Glu Val Val Asp Ser Ser Gly
    50                  55                  60
Ser Ala Ile Pro Leu Leu Leu Arg Glu Leu Asn Arg Phe Asp Gly Ser
65                  70                  75                  80
Gly Pro Tyr Ser Thr Leu Ser Ser Pro Gln Leu Gly Arg Ser Thr Tyr
                85                  90                  95
Met Glu Thr Tyr Val Tyr Phe Tyr Arg Ser His Lys Thr Gln Val Leu
            100                 105                 110
Ser Ser Tyr Val Tyr Asn Asp Glu Asp Asp Val Phe Ala Arg Glu Pro
        115                 120                 125
Phe Val Ala Gln Phe Ser Leu Pro Ser Asn Val Leu Pro Ser Leu Val
    130                 135                 140
Leu Val Pro Leu His Thr Thr Pro Lys Ala Val Glu Lys Glu Leu Asn
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145                 150                 155                 160
Ala Leu Tyr Asp Val Phe Leu Glu Val Ser Gln His Trp Gln Ser Lys
                165                 170                 175
Asp Val Ile Leu Leu Gly Asp Phe Asn Ala Asp Cys Ala Ser Leu Thr
            180                 185                 190
Lys Lys Arg Leu Asp Lys Leu Glu Leu Arg Thr Glu Pro Gly Phe His
        195                 200                 205
Trp Tyr Ile Ala Asp Gly Glu Asp Thr Thr Val Arg Ala Ser Thr His
    210                 215                 220
Cys Thr Tyr Asp Arg Val Val Leu His Gly Glu Arg Cys Arg Ser Leu
225                 230                 235                 240
Leu His Thr Ala Ala Ala Phe Asp Phe Pro Thr Ser Phe Gln Leu Thr
                245                 250                 255
Glu Glu Glu Ala Leu Asn Ile Ser Asp His Tyr Pro Val Glu Val Glu
            260                 265                 270
Leu Lys Leu Ser Gln Ala His Ser Val Gln Pro Leu Ser Leu Thr Val
        275                 280                 285
Leu Leu Leu Leu Ser Leu Leu Ser Pro Gln Leu Cys Pro Ala Ala
    290                 295                 300
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INTERNATIONAL SEARCH REPORT

Interna Application No
PCT/ 03/00668

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/46 A61K31/726 A61K31/727 A61K31/728 A61K31/737 A61P11/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 10053 A (BAXTER INT) 12 March 1998 (1998-03-12) claims 1,4	25,33,34
X	US 6 235 725 B1 (AHMED TAHIR) 22 May 2001 (2001-05-22)	29,33,34
Y	column 7, paragraphs 1,5 column 8, line 57 -column 9, line 4 column 10, line 40-60	4-7
X	GB 1 560 463 A (RIKER LABORATORIES INC) 6 February 1980 (1980-02-06) page 1, line 13-22	29,33-35
X	US 5 633 003 A (CANTOR JEROME O) 27 May 1997 (1997-05-27)	29,33-35
Y	column 2, line 65; claims	4-7

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 July 2003	09/07/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Deck, A

INTERNATIONAL SEARCH REPORT

Interna pplication No
PCT/ GB 03/00668

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 074 260 A (SEIKAGAKU KOGYO CO LTD) 7 February 2001 (2001-02-07)	29,33-35
Y	page 6, paragraph 41; claims	4-7
X	WO 95 23613 A (GENENTECH INC) 8 September 1995 (1995-09-08)	30,33-35
Y	page 10, line 30 -page 11, line 24	1-36
Y	WO 01 15672 A (KING MALCOLM ;GOVERNORS OF THE UNIVERSITY OF (CA)) 8 March 2001 (2001-03-08) page 8, line 2-10	1-36

INTERNATIONAL SEARCH REPORT

Inb nal application No.
rcT/GB 03/00668

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 28 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Internal application No
PCT/GB 03/00668

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		WO 0115672 A2	08-03-2001
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